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(57) Abstract

Isolated CT-1, isolated DNA encoding CT-1, and recombinant or synthetic methods of preparing CT-1 are disclosed. CT-1 is shown to bind to and activate the receptor, LIFRβ. These CT-1 molecules are shown to influence hypertrophic activity, neurological activity, and other activities associated with receptor LIFRβ. Accordingly, these compounds or their antagonists may be used for treatment of heart failure, arrhythmic disorders, inotropic disorders, neurological disorders, and other disorders associated with the LIFRβ.

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CARDIOTROPHIN AND USES THEREFOR

TECHNICAL FIELD

This application relates to a cardiac hypertrophy factor (also known as CT-1) for modulating cardiac function in the treatment of heart failure, for modulating neural function in the treatment of neurological disorders, and for treatment of a variety of other disorders related to a CT-1 receptor, particularly the LIFRβ.

BACKGROUND

Heart failure affects approximately three million Americans, developing in about 400,000 each year. It is currently one of the leading admission diagnoses in the U.S. Recent advances in the management of acute cardiac diseases, including acute myocardial infarction, are resulting in an expanding patient population that will eventually develop chronic heart failure.

Current therapy for heart failure is primarily directed to using angiotensin-converting enzyme (ACE) inhibitors and diuretics. While prolonging survival in the setting of heart failure, ACE inhibitors appear to slow the progression towards end-stage heart failure, and substantial numbers of patients on ACE inhibitors have functional class III heart failure. Moreover, ACE inhibitors consistently appear unable to relieve symptoms in more than 60% of heart failure patients and reduce mortality of heart failure only by approximately 15-20%. Heart transplantation is limited by the availability of donor hearts. Further, with the exception of digoxin, the chronic administration of positive inotropic agents has not resulted in a useful drug without accompanying adverse side effects, such as increased arrhythmogenesis, sudden death, or other deleterious side effects related to survival. These deficiencies in current therapy suggest the need for additional therapeutic approaches.

A wide body of data suggests that pathological hypertrophy of cardiac muscle in the setting of heart failure can be deleterious, characterized by dilation of the ventricular chamber, an increase in wall tension/stress, an increase in the length vs. width of cardiac muscle cells, and an accompanying decrease in cardiac performance and function. Studies have shown that the activation of physiological or compensatory hypertrophy can be beneficial in the setting of heart failure. In fact, the effects of ACE inhibitors have been purported not only to unload the heart, but also to inhibit the pathological hypertrophic response that has been presumed to be linked to the localized renin-angiotensin system within the myocardium.

Cardiac muscle hypertrophy is an important adaptive response of the heart to injury or to an increased demand for cardiac output. This hypertrophic response is characterized by the reactivation of genes normally expressed during fetal heart development and by the accumulation of sarcomeric proteins in the absence of DNA replication or cell division (Rockman *et al.*, *Circulation*, 87:VII14-VII21 (1993); Chien, *FASEB J.*, 5:3037-3046 (1991); Shubeita *et al.*, *J. Biol. Chem.*, 265:20555-20562 (1990)).

On a molecular biology level, the heart functions as a syncytium of myocytes and surrounding support cells, called non-myocytes. While non-myocytes are primarily fibroblast/mesenchymal cells, they also include endothelial and smooth muscle cells. Indeed, although myocytes make up most of the adult myocardial mass, they represent only about 30% of the total cell numbers present in heart. Because of their close relationship with cardiac myocytes *in vivo*, non-myocytes are capable of influencing myocyte growth and/or development. This interaction may be mediated directly through cell-cell contact or indirectly via production of a paracrine factor. Such association *in vivo* is important since both non-myocyte numbers and the extracellular matrix with which

they interact are increased in myocardial hypertrophy and in response to injury and infarction. These changes are associated with abnormal myocardial function.

Cardiac myocytes are unable to divide shortly after birth. Further growth occurs through hypertrophy of the individual cells. Cell culture models of myocyte hypertrophy have been developed to understand better the mechanisms for cardiac myocyte hypertrophy. Simpson *et al.*, *Circ. Res.*, 51:787-801 (1982); Chien *et al.*, *FASEB J.*, 5:3037-3046 (1991). Most studies of heart myocytes in culture are designed to minimize contamination by non-myocytes. See, for example, Simpson *et al.*, *Cir. Cres.*, 50:101-116 (1982); Libby, *J. Mol. Cell. Cardiol.*, 16:803-811 (1984); Iwaki *et al.*, *J. Biol. Chem.*, 265:13809-13817 (1990).

Shubaita *et al.*, *J. Biol. Chem.*, 265:20555-20562 (1990) documented the utility of a culture model to identify peptide-derived growth factors such as endothelin-1 that can activate a hypertrophic response. Long *et al.*, *Cell Regulation*, 2:1081-1095 (1991) investigated the effect of the cardiac non-myocytes on cardiac myocyte growth in culture. Myocyte hypertrophic growth was stimulated in high-density cultures with increased numbers of non-myocytes and in co-cultures with increased numbers of non-myocytes. This effect of non-myocytes on myocyte size could be reproduced by serum-free medium conditioned by non-myocyte cultures. The major myocyte growth-promoting activity in the cultures was heparin binding. The properties of this growth factor were compared to various growth factors known to be present in myocardium, including fibroblast growth factor (FGF), platelet derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta1 (TGF-β1). The growth factor of Long *et al.* was found to be larger than these other known growth factors and to have a different heparin-Sepharose elution profile from that of all these growth factors except PDGF. Further, it was not neutralized by a PDGF-specific antibody. The authors proposed that it defines a paracrine relationship important for cardiac muscle cell growth and development.

Not only is there a need for an improvement in the therapy of heart failure such as congestive heart failure, but there is also a need to offer effective treatment for neurological disorders. Neurotrophic factors such as insulin-like growth factors, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, -4, and -5, and ciliary neurotrophic factor have been proposed as potential means for enhancing neuronal survival, for example, as a treatment for neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, stroke, epilepsy, Huntington's disease, Parkinson's disease, and peripheral neuropathy. It would be desirable to provide an additional therapy for this purpose.

In addition, there is a need for identification of and improvement in the therapy of diseases for which cytokines, their antagonists or agonists play a role. The IL-6 family of cytokines (IL-6/LIF/CNTF/OSM/IL-11) has a wide range of growth and differentiation activities on many cell types including those from the blood, liver, and nervous system (Akira *et al.*, *Adv. Immunol.*, 54:1-78 (1993); Kishimoto *et al.*, *Science*, 258:593-597 (1992)). The biological effects induced by IL-6 and related proteins are mediated by a family of structurally similar cell surface receptors, the cytokine receptor family, that includes the receptors for growth hormone and prolactin as well as for many cytokines (Cosman *et al.*, *Trends Biochem. Sci.*, 15:265-270 (1990); Miyajima *et al.*, *Ann. Rev. Immunol.*, 10:295-331 (1992); Taga et al., *FASEB J*, 6:3387-3396 (1992); Bazan, *Immunol. Today*, 11:350-354 (1990)). The IL-6 receptor subfamily is composed of multi-subunit complexes that share a common signaling subunit, gp130 (Davis *et al.*, *Curr. Opin. Cell Biol.*, 5:281-285 (1993); Stahl *et al.*, *Cell*, 74:587-590 (1993); Kishimoto *et al.*, *Cell*, 76:253-262 (1994)). Some members of the IL-6 cytokine family (IL-6 and IL-11)

induce the homodimerization of gp130 (Murakami *et al.*, *Science*, 260:1808-1810 (1993); Hilton et al., *EMBO J.*, 13:4765-4775 (1994)), while others (LIF, OSM and CNTF) induce gp130 heterodimer formation with the 190 kDa LIF receptor (Davis *et al.*, *Science*, 260:1805-1808 (1993)). Following dimerization of the signaling components, these receptors induce a number of intracellular signaling events including activation of the transcription factor, NF-IL6, probably via the ras-MAP kinase cascade (Kishimoto *et al.*, *Cell*, 76:253-262 (1994)), and activation of the Jak/STAT signaling pathway (Darnell *et al.*, *Science*, 264:1415-1421 (1994)). The latter pathway includes the tyrosine phosphorylation and activation of the intracellular tyrosine kinases, Jak1, Jak2, and Tyk2 (Lütticken *et al.*, *Science*, 263:89-92 (1994); Stahl *et al.*, *Science*, 263:92-95 (1994); Yin *et al.*, *Exp. Hematol.*, 22:467-472 (1994); Narazaki *et al.*, *Proc. Natl. Acad. USA*, 91:2285-2289 (1994)) and of the transcription factors, STAT1 and STAT3 (Lütticken *et al.*, *Science*, 263:89-92 (1994); Zhong *et al.*, *Science*, 264:95-98 (1994); Akira *et al.*, *Cell*, 77:63-71 (1994)).

Accordingly, it is an object of the present invention to provide an improved therapy for the prevention and/or treatment of heart failure such as congestive heart failure, particularly the promotion of physiological forms of hypertrophy or inhibition of pathological forms of hypertrophy, for the prevention and/or treatment of neurological disorders such as peripheral neuropathy, and for the prevention and treatment of disorders in which cytokines, particularly the IL-6/LIF/CNTF/OSM/IL-11 cytokine family, their antagonists, their agonists, or their receptors play a role.

These and other objects of the invention will be apparent to the ordinarily skilled artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

An *in vitro* neonatal rat heart hypertrophy assay has been developed that allows for expression cloning and protein purification of the cardiac hypertrophy factor (referred to as CHF, more preferably cardiotrophin-1 or CT-1) disclosed herein. The assay capacity of 1000 single samples a week coupled with the small sample size requirement of 100 µL or less has enabled expression cloning and protein purification that would have been impossible using the currently published methods. Hence, in one embodiment, the invention provides a method for assaying a test sample for hypertrophic activity comprising:

(a) plating 96-well plates with a suspension of myocytes at a cell density of about 7.5×10^4 cells per mL in Dulbecco's modified Eagle's medium (D-MEM)/F-12 medium comprising insulin, transferrin, and aprotinin;

(b) culturing the cells;

(c) adding the test sample (such as one suspected of containing a CT-1) to the cultured cells;

(d) culturing the cells with the test sample; and

(e) determining if the test sample has hypertrophic activity.

Besides the assay, the invention provides isolated CT-1 polypeptide. This CT-1 polypeptide is preferably substantially homogeneous, may be glycosylated or unglycosylated, and may be selected from the group consisting of the native sequence polypeptide, a fragment polypeptide, a variant polypeptide, and a chimeric polypeptide. Additionally, the CT-1 polypeptide may be selected from the group consisting of the polypeptide that is isolated from a mammal, the polypeptide that is made by recombinant means, and the

polypeptide that is made by synthetic means. Further, this CT-1 polypeptide may be selected from the group consisting of the polypeptide that is human and the polypeptide that is non-immunogenic in a human.

In another aspect, the isolated CT-1 polypeptide shares at least 75% amino acid sequence identity with the translated CT-1 sequence shown in Fig. 1. In a further aspect, the polypeptide is the mature human CT-1 having the translated CT-1 sequence shown in Fig. 5.

In a still further aspect, the invention provides an isolated polypeptide encoded by a nucleic acid having a sequence that hybridizes under moderately stringent conditions to the nucleic acid sequence provided in Fig. 1. Preferably, this polypeptide is biologically active.

In another aspect, the invention provides a chimera comprising CT-1 fused to a heterologous polypeptide.

In a still further aspect, the invention provides a composition comprising biologically active CT-1 and a pharmaceutically acceptable carrier or comprising biologically active CT-1 fused to an immunogenic polypeptide.

In yet another aspect, the invention provides an isolated antibody that is capable of binding CT-1 and a method for detecting CT-1 *in vitro* or *in vivo* comprising contacting the antibody with a sample or cell suspected of containing CT-1 and detecting if binding has occurred, as with an ELISA.

In still another aspect, the invention provides a method for purifying CT-1 comprising passing a mixture of CT-1 over a column to which is bound the antibodies and recovering the fraction containing CT-1.

In other aspects, the invention comprises an isolated nucleic acid molecule encoding CT-1, a vector comprising the nucleic acid molecule, preferably an expression vector comprising the nucleic acid molecule operably linked to control sequences recognized by a host cell transformed with the vector, a host cell comprising the nucleic acid molecule, including mammalian and bacterial host cells, and a method of using a nucleic acid molecule encoding CT-1 to effect the production of CT-1, comprising culturing a host cell comprising the nucleic acid molecule. Preferably the host cell is transfected to express CT-1 nucleic acid and the CT-1 is recovered from the host cell culture, and if secreted, recovered from the culture medium.

In additional aspects, the invention provides an isolated nucleic acid molecule comprising the open reading frame nucleic acid sequence shown in Fig. 1 or Fig. 5. The invention also provides an isolated nucleic acid molecule selected from the group consisting of:

(a) a cDNA clone comprising the nucleotide sequence of the coding region of the CT-1 gene shown in Figure 1 or Figure 5;

(b) a DNA sequence capable of hybridizing under stringent conditions to a clone of (a); and

(c) a genetic variant of any of the DNA sequences of (a) and (b) which encodes a polypeptide possessing a biological property of a native CT-1 polypeptide.

The invention also provides an isolated DNA molecule having a sequence capable of hybridizing to the DNA sequence provided in Fig. 1 or Fig. 5 under moderately stringent conditions, wherein the DNA molecule encodes a biologically active CT-1 polypeptide, excluding rat CT-1.

In yet another aspect, a method is provided of determining the presence of a CT-1 nucleic acid molecule in a test sample comprising contacting the CT-1 nucleic acid molecule with the test sample and determining

whether hybridization has occurred, or comprising hybridizing the CT-1 nucleic acid molecule to a test sample nucleic acid and determining the presence of CT-1 nucleic acid.

In still another aspect, the invention provides a method of amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase chain reaction in the test sample with the CT-1 nucleic acid molecule.

In a still further aspect, the invention provides a CT-1 antagonist and a method of identifying such antagonist comprising using cell supernatants as the test sample in the hypertrophy assay as described above and screening for molecules that antagonize the hypertrophic activity of a CT-1 demonstrated in such assay.

In a still further aspect, the invention provides a method for treating a mammal having or at risk for heart failure, an inotropic disorder, or an arrhythmic disorder comprising administering to a mammal in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising the CT-1 or a CT-1 antagonist in a pharmaceutically acceptable carrier.

The invention also provides a method for treating a mammal having or at risk for a neurological disorder comprising administering to a mammal in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising the CT-1 in a pharmaceutically acceptable carrier.

The invention also provides a method for treating a mammal having or at risk for a disorder in which cytokines, particularly the IL-6/LIF/CNTF/OSM/IL-11 cytokine family, more preferably LIF and OSM, more preferably LIF, their antagonists or their agonists, and most preferably a LIF-Receptor β subunit that interacts with gp130, play a role. The methods comprise administering to a mammal in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising CT-1, its antagonist, or its agonist, in a pharmaceutically acceptable carrier. In a most preferred embodiment the disorders involve a pathway regulated or induced by the activation of LIFRβ by CT-1 binding and subsequent interaction with gp130.

In a still further aspect, the invention provides a CT-1 antagonist and a method of identifying such antagonist comprising using cell supernatants or purified or synthetic compounds as the test sample in an assay in which CT-1 has a demonstrated biological activity, receptor binding activity, or signaling pathway induction activity, preferably in a microassay, and screening for molecules that antagonize the activity of a CT-1 demonstrated in such an assay.

In additional embodiments, the invention supplies a method of identifying a receptor for CT-1 comprising using labeled CT-1, preferably radiolabeled CT-1, in a cellular receptor assay, allowing the CT-1 to bind to cells, or using the labeled CT-1 to pan for cells that contain the receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict the nucleotide sequence (sense and anti-sense strands) (SEQ ID NOS: 1 and 2) and deduced amino acid sequence (SEQ ID NO: 3) of a mouse CT-1 DNA clone. The underlined complementary nucleotides at position 27 show the start of another mouse CT-1 clone used to obtain the full-length clone.

Figure 2 aligns the translated amino acid sequence of the mouse CT-1 clone (chf.781) (SEQ ID NO: 3) with the amino acid sequence of human ciliary neurotrophic factor (humcntf) (SEQ ID NO: 4) to show the extent of sequence identity.

Figure 3 shows a graph of atrial natriuretic peptide (ANP) release for phenylephrine (standard curve) and transfections into 293 cells in a neonatal cardiac hypertrophy assay.

Figure 4 shows a graph of survival of live ciliary ganglion neurons (measured by cell count) as a function of either the ciliary neutrotrophic factor (CNTF) standard (in ng/mL) or the transfected 293 conditioned medium (in fraction of assay volume), using a CNTF standard (circles), medium from a CT-1 DNA transfection of 293 cells (triangles), and medium from a control DNA transfection of 293 cells (squares).

Figures 5A and 5B depict the nucleotide sequence (sense and anti-sense strands) (SEQ ID NOS: 6 and 7) and deduced amino acid sequence (SEQ ID NO: 8) of a human CT-1 DNA clone.

Figure 6 aligns the translated amino acid sequence of the human CT-1 clone (humct1) (SEQ ID NO: 8) with the translated amino acid sequence of the mouse CT-1 clone (chf.781) (SEQ ID NO: 3) to show the extent of sequence identity.

Figures 7A and 7B depict activity of CT-1 in hematopoietic cell assays. The induction by the human (*h*) or mouse (*m*) cytokines was performed as described in the Example VI, Materials and Methods. Figure 7A shows stimulation of ^{3}H-thymidine incorporation in the mouse hybridoma cell line, B9, with an EC_{50} [IL-6] = 0.13 (± 0.03) nM. Figure 7B shows inhibition of ^{3}H-thymidine incorporation in the mouse myeloid leukemia cell line, M1, with an EC_{50} [CT-1] = 0.0076 (± 0.0006) nM. EC_{50} [LIF] = 0.048 (± 0.004) nM.

Figures 8A, 8B, and 8C depict activity of CT-1 in neuronal cell assays. The induction by mouse (*m*) or rat (*r*) cytokines was performed as described in Example VI, Materials and Methods. Figure 8A shows the switch in transmitter phenotype of rat sympathetic neurons. Tyrosine hydroxylase (*TH*) and choline acetyltransferase (*ChAT*) activities were determined in duplicate. Figure 8B shows survival of rat dopaminergic neurons. Plotted are the average and standard deviation of triplicate determinations. Figure 8C shows survival of chick ciliary neurons with an EC_{50} [CT-1] = 10 (± 8.2) nM and EC_{50} [CNTF] = 0.0074 (± 0.0049) nM.

Figure 9 depicts activity of CT-1 in embryonic stem cells development. Mouse embryonic stem cells were cultured in the presence of the mouse (*m*) cytokines as described in Example VI, Materials and Methods.

Figures 10A, 10B, 10C and 10D depict binding and cross-competition of CT-1 and LIF to mouse M1 cells. Assays contained 0.047 nM ^{125}I-mouse CT-1 (*^{125}I-mCT-1*) and unlabeled mouse (*m*) CT-1, Figure 10A, or unlabeled LIF, Figure 10B; or 0.042 nM ^{125}I-mouse LIF (*^{125}I-mLIF*) and unlabeled CT-1, Figure 10C, or LIF, Figure 10D. Shown are competition and Scatchard (*insert*) plots of the data. For the labeled CT-1 binding, K_d [CT-1] = 0.61 (± 0.11) nM, 1500 (± 220) sites/cell; K_d [LIF] = 0.19 (± 0.05) nM, 1800 (± 150) sites/cell. For labeled LIF binding, K_d [CT-1] = 0.83 (± 0.13) nM, 1300 (± 80) sites/cell; K_d [LIF] = 0.26 (± 0.10) nM, 1200 (± 300) sites/cell.

Figure 11 depicts cross-linking of CT-1 and LIF to M1 Cells. ^{125}I-mouse CT-1 (*^{125}I-mCT-1*) or ^{125}I-mouse LIF (*^{125}I-mLIF*) were bound and cross-linked to M1 cells in the absence (*None*) or presence of a 100 fold excess of the indicated mouse (*m*) cytokine, and the reaction products analyzed by SDS gel electrophoresis. The mobility of molecular weight standards is indicated.

Figures 12A depicts inhibition of CT-1 binding to M1 cells by an anti-gp130 monoclonal antibody. Assays contained 0.12 nM ^{125}I-mouse CT-1 and antibodies as indicated. For the anti-gp130 antibody, EC_{50} = 44 (± 8) nM. Figure 12B depicts electrophoretic mobility shift of the DNA element SIE induced by CT-1 binding to M1 cells. M1 cells were incubated without (-) or with (+) 5 nM mouse (*m*) CT-1 or LIF, lysed, and

the cell extract assayed for binding to the DNA element SIE as described in the Materials and Methods. Binding specificity was determined by the addition of unlabeled SIE DNA (*Cold Oligo*). The specific DNA complex is indicated (*arrow*).

Figure 13A and 13B depict binding and cross-competition of CT-1 and LIF to rat primary cardiac myocytes. Duplicate assays contained either 0.047 nM ^{125}I-mouse CT-1 (*^{125}I-mCT-1*) or 0.042 nM ^{125}I-mouse LIF (*^{125}I-mLIF*) and unlabeled mouse (*m*) CT-1 or LIF as indicated.

Figures 14A, 14B, 14C and 14D depict binding of CT-1 to purified, soluble LIF receptor and gp130. Figures 14A-C show per cent binding of ^{125}I-mouse CT-1 (0.089 nM) to soluble mouse LIF receptor (*smLIFR*) and soluble mouse gp130 (*smgp130*) in the absence (-) or presence (+) of 164 nM unlabeled mouse CT-1 (*mCT-1*). Figure 14A depicts binding to increasing concentrations soluble LIF receptor alone: FIgure 14B depicts binding to increasing concentrations of soluble gp130 alone; Figure 14C depicts binding at one soluble LIF receptor concentration with increasing concentrations of soluble gp130. Plotted is the average and half the difference of duplicate determinations. The results for 0.84 nM soluble LIF receptor are shown twice for clarity. Figure 14D depicts competition binding of ^{125}I-mouse CT-1 (0.089 nM) to the soluble LIF receptor (2.8 nM) with increasing concentrations of unlabeled CT-1. K_d [CT-1] = 1.9 (± 0.2) nM.

Figures 15A and 15B depict similarity of IL-6 family ligands and subunit structure of their receptors. Figure 15A shows per cent amino acid identity of the mature form of the IL-6 family ligands: (*m*) mouse, (*h*) human, (*c*) chicken. The bottom row gives the per cent identity of the cytokine to its human homologue. Shown in bold are the percentages greater than 40 %. Figure 15B is a diagram of the IL-6 family receptors. The subunit stoichiometry of the various complexes is not known in most cases, although recent work has led to a conclusion that the IL-6 receptor complex is a hexamer containing two IL-6 molecules, two IL-6 receptors, and two gp130 signaling subunits. Ward *et al.*, *J. Biol. Chem.*, 269:23286-23289 (1994).

Figure 16 depicts alignment of the protein sequence of human CT-1, LIF and CNTF. Encoded amino acid sequence of human CT-1 (hCT-1) aligned with that of human LIF (hLIF) and human CNTF (hCNTF). Overlining indicates the location of four amphipathic helices based on their proposed locations in CNTF (Bazan, *Neuron*, 7:197-208 (1991)).

Figures 17A and 17B depict the competition for the binding of human LIF to mouse M1 or human Hela cell. For Figure 17A 125I-human LIF was bound in duplicate to M1 (5 million cells per reaction) in the presence of the indicated competitors. For Figure 17B 125I-human LIF was bound in duplicate to Hela cells (2.5 million per reaction) in the presence of the indicated competitors. CM is conditioned medium from 293 cells transfected with human CT-1.

Figure 18 depicts the binding of mouse CT-1 to human Hela cells. Duplicate assays containing 0.23 nM 125I-mouse-CT-1 and 9 million cells were performed as described in the Examples. The insert is a Scatchard plot of the data. Kd=0.75 (+/-0.15) nM, 860(+/-130 sites per cell).

Figure 19 depicts the competition for the binding of human OSM to human WI-26 cells. 125I-human OSM was bound in duplicate to WI-26 VA4 cells (2.4 million cells per reaction) in the presence of the indicated competitors as described in the Examples.

Figure 20 depicts expression of CT-1 in human tissues. Northern blots containing polyA+RNA from the indicated tissues were hybridized with a human CT-1 cDNA probe as described in the Examples.

Figure 21 is a schematic depicting several biological activities of CT-1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims:

"CHF" (or "cardiac hypertrophy factor" or "cardiotrophin" or "cardiotrophin-1" or "CT-1") is defined herein to be any polypeptide sequence that possesses at least one biological property (as defined below) of a naturally occurring polypeptide comprising the polypeptide sequence of Fig. 1 or the human equivalent thereof shown in Fig. 5. It does not include the rat homolog of CT-1, *i.e.*, CT-1 from the rat species. This definition encompasses not only the polypeptide isolated from a native CT-1 source such as murine embryoid bodies described herein or from another source, such as another animal species except rat, including humans, but also the polypeptide prepared by recombinant or synthetic methods. It also includes variant forms including functional derivatives, alleles, isoforms and analogues thereof.

A "CT-1 fragment" is a portion of a naturally occurring mature full-length CT-1 sequence having one or more amino acid residues or carbohydrate units deleted. The deleted amino acid residue(s) may occur anywhere in the polypeptide, including at either the N-terminal or C-terminal end or internally. The fragment will share at least one biological property in common with CT-1. CT-1 fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of the CT-1 isolated from a mammal including the CT-1 isolated from murine embryoid bodies or the human CT-1.

"CT-1 variants" or "CT-1 sequence variants" as defined herein mean biologically active CT-1s as defined below having less than 100% sequence identity with the CT-1 isolated from recombinant cell culture or from murine embryoid bodies having the deduced sequence described in Fig. 1, or with the human equivalent described in Fig. 5. Ordinarily, a biologically active CT-1 variant will have an amino acid sequence having at least about 70% amino acid sequence identity with the CT-1 isolated from murine embryoid bodies or the mature human CT-1 (see Figs. 1 and 5), preferably at least about 75%, more preferably at least about 80%, still more preferably at least about 85%, even more preferably at least about 90%, and most preferably at least about 95%.

A "chimeric CT-1" is a polypeptide comprising full-length CT-1 or one or more fragments thereof fused or bonded to a second protein or one or more fragments thereof. The chimera will share at least one biological property in common with CT-1. The second protein will typically be a cytokine, growth factor, or hormone such as growth hormone, IGF-I, or a neurotrophic factor such as CNTF, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-5 (NT-5), NT-6, or the like.

"Isolated CT-1", "highly purified CT-1" and "substantially homogeneous CT-1" are used interchangeably and mean a CT-1 that has been purified from a CT-1 source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-

reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means less than about 5% contamination with other source proteins.

"Biological property" when used in conjunction with either "CT-1" or "isolated CT-1" means having myocardiotrophic, inotropic, anti-arrhythmic, or neurotrophic activity or having an *in vivo* effector or antigenic function or activity that is directly or indirectly caused or performed by a CT-1 (whether in its native or denatured conformation) or a fragment thereof. Effector functions include receptor binding and any carrier binding activity, agonism or antagonism of CT-1, especially transduction of a proliferative signal including replication, DNA regulatory function, modulation of the biological activity of other growth factors, receptor activation, deactivation, up- or down-regulation, cell growth or differentiation, and the like. However, effector functions do not include possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against native CT-1.

An "antigenic function" means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the native CT-1 whose sequence is shown in Fig. 1 or another mammalian native CT-1, including the human homolog whose sequence is shown in Fig. 5. The principal antigenic function of a CT-1 polypeptide is that it binds with an affinity of at least about 10^6 L/mole to an antibody raised against CT-1 isolated from mouse embryoid bodies or a human homolog thereof. Ordinarily, the polypeptide binds with an affinity of at least about 10^7 L/mole. Most preferably, the antigenically active CT-1 polypeptide is a polypeptide that binds to an antibody raised against CT-1 having one of the above-described effector functions. The antibodies used to define "biologically activity" are rabbit polyclonal antibodies raised by formulating the CT-1 isolated from recombinant cell culture or embryoid bodies in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of the anti-CT-1 antibody plateaus.

"Biologically active" when used in conjunction with either "CT-1" or "isolated CT-1" mean a CT-1 polypeptide that exhibits hypertrophic, inotropic, anti-arrhythmic, or neurotrophic activity or shares an effector function of CT-1 isolated from murine embryoid bodies or produced in recombinant cell culture described herein, and that may (but need not) in addition possess an antigenic function. One principal effector function of CT-1 or CT-1 polypeptide herein is influencing cardiac growth or hypertrophy activity, as measured, *e.g.*, by atrial natriuretic peptide (ANP) release or by the myocyte hypertrophy assay described herein using a specific plating medium and plating density, and preferably using crystal violet stain for readout. The desired function of a CT-1 (or CT-1 antagonist) is to increase physiological (beneficial) forms of hypertrophy and decrease pathological hypertrophy. In addition, the CT-1 herein is expected to display anti-arrhythmic function by promoting a more normal electrophysiological phenotype. Another principal effector function of CT-1 or CT-1 polypeptide herein is stimulating the proliferation of chick ciliary ganglion neurons in an assay for CNTF activity.

Antigenically active CT-1 is defined as a polypeptide that possesses an antigenic function of CT-1 and that may (but need not) in addition possess an effector function.

In preferred embodiments, antigenically active CT-1 is a polypeptide that binds with an affinity of at least about 10^6 L/mole to an antibody capable of binding CT-1. Ordinarily, the polypeptide binds with an affinity of at least about 10^7 L/mole. Isolated antibody capable of binding CT-1 is an antibody that is identified and separated from a component of the natural environment in which it may be present. Most preferably, the

antigenically active CT-1 is a polypeptide that binds to an antibody capable of binding CT-1 in its native conformation. CT-1 in its native conformation is CT-1 as found in nature that has not been denatured by chaotropic agents, heat, or other treatment that substantially modifies the three-dimensional structure of CT-1 as determined, for example, by migration on non-reducing, non-denaturing sizing gels. Antibody used in this determination is rabbit polyclonal antibody raised by formulating native CT-1 from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-CT-1 antibody plateaus.

"Percent amino acid sequence identity" with respect to the CT-1 sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the CT-1 sequence isolated from murine embryoid bodies having the deduced amino acid sequence described in Fig. 1 or the deduced human CT-1 amino acid sequence described in Fig. 5, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the CT-1 sequence shall be construed as affecting sequence identity or homology. Thus, exemplary biologically active CT-1 polypeptides considered to have identical sequences include prepro-CT-1, pro-CT-1, and mature CT-1.

"CT-1 microsequencing" may be accomplished by any appropriate standard procedure provided the procedure is sensitive enough. In one such method, highly purified polypeptide obtained from SDS gels or from a final HPLC step is sequenced directly by automated Edman (phenyl isothiocyanate) degradation using a model 470A Applied Biosystems gas-phase sequencer equipped with a 120A phenylthiohydantoin (PTH) amino acid analyzer. Additionally, CT-1 fragments prepared by chemical (*e.g.*, CNBr, hydroxylamine, or 2-nitro-5-thiocyanobenzoate) or enzymatic (*e.g.*, trypsin, clostripain, or staphylococcal protease) digestion followed by fragment purification (*e.g.*, HPLC) may be similarly sequenced. PTH amino acids are analyzed using the ChromPerfect™ data system (Justice Innovations, Palo Alto, CA). Sequence interpretation is performed on a VAX 11/785 Digital Equipment Co. computer as described by Henzel *et al.*, *J. Chromatography*, 404:41-52 (1987). Optionally, aliquots of HPLC fractions may be electrophoresed on 5-20% SDS-PAGE, electrotransferred to a PVDF membrane (ProBlott, AIB, Foster City, CA) and stained with Coomassie Brilliant Blue. Matsurdiara, *J. Biol. Chem.*, 262:10035-10038 (1987). A specific protein identified by the stain is excised from the blot and N-terminal sequencing is carried out with the gas-phase sequenator described above. For internal protein sequences, HPLC fractions are dried under vacuum (SpeedVac), resuspended in appropriate buffers, and digested with cyanogen bromide, the Lys-specific enzyme Lys-C (Wako Chemicals, Richmond, VA), or Asp-N (Boehringer Mannheim, Indianapolis, IN). After digestion, the resultant peptides are sequenced as a mixture or after HPLC resolution on a C4 column developed with a propanol gradient in 0.1% trifluoroacetic acid (TFA) prior to gas-phase sequencing.

"Isolated CT-1 nucleic acid" is RNA or DNA containing greater than 16 and preferably 20 or more sequential nucleotide bases that encodes biologically active CT-1 or a fragment thereof, is complementary to the RNA or DNA, or hybridizes to the RNA or DNA and remains stably bound under moderate to stringent conditions. This RNA or DNA is free from at least one contaminating source nucleic acid with which it is normally associated in the natural source and preferably substantially free of any other mammalian RNA or

DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is present in the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell. An example of isolated CT-1 nucleic acid is RNA or DNA that encodes a biologically active CT-1 sharing at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the murine CT-1 or with the human CT-1.

"Control sequences" when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Exogenous" when referring to an element means a nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

"Cell," "cell line," and "cell culture" are used interchangeably herein and such designations include all progeny of a cell or cell line. Thus, for example, terms like "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are autonomously replicating circular DNA molecules possessing independent origins of replication and are designated herein by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein either are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accordance with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" when referring to DNA means catalytic cleavage of internal phosphodiester bonds of DNA with an enzyme that acts only at certain locations or sites in the DNA sequence. Such enzymes are called "restriction endonucleases." Each restriction endonuclease recognizes a specific DNA sequence called a "restriction site" that exhibits two-fold symmetry. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the

enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μL of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation for about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in sections 1.56-1.61 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, *Nucleic Acids Res.*, 9:6103-6114 (1981) and Goeddel *et al.*, *Nucleic Acids Res.*, 8:4057 (1980).

"Southern analysis" or "Southern blotting" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or a DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook *et al.*, *supra*.

"Northern analysis" or "Northern blotting" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

"Ligation" is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol

precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 µg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large- and small-scale plasmid preparations described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid-phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, *Nucl. Acids Res.*, 14:5399-5407 (1986). Further methods include the polymerase chain reaction defined below and other autoprimer methods and oligonucleotide syntheses on solid supports. All of these methods are described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, 28:716-734 (1989). These methods are used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides are then purified on polyacrylamide gels.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, *etc.* See generally Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 51:263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

"Stringent conditions" are those that (Chien *et al.*, *Annu. Rev. Physiol.*, 55:77-95 (1993)) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% $NaDodSO_4$ (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

"Moderately stringent conditions" are described in Sambrook *et al.*, *supra*, and include the use of a washing solution and hybridization conditions (*e.g.*, temperature, ionic strength, and %SDS) less stringent than described above. An example of moderately stringent conditions is a condition such as overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, *etc.*, as necessary to accommodate factors such as probe length and the like.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas. "Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains (Clothia *et al.*, *J. Mol. Biol.*, 186:651-663 (1985); Novotny *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:4592-4596 (1985)).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen- binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3, IgG-4, IgA-1, and IgA-2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies) and antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-CT-1 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. (See, e.g. Cabilly, *et al.*, U.S. Pat. No. 4,816,567; Mage *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp.79-97 (Marcel Dekker, Inc., New York, 1987).)

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (Cabilly *et al.*, *supra*). The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, *supra*; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones *et al.*, *Nature*, 321:522-525 (1986); Reichmann *et al.*, *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

"Non-immunogenic in a human" means that upon contacting the polypeptide in a pharmaceutically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide is demonstratable upon the second administration of the polypeptide after an appropriate latent period (*e.g.*, 8 to 14 days).

"Neurological disorder" refers to a disorder of neurons, including both peripheral neurons and neurons from the central nervous system. Examples of such disorders include all neurodegenerative diseases, such as peripheral neuropathies (motor and sensory), amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, stroke, Huntington's disease, epilepsy, and ophthalmologic diseases such as those involving the retina, *e.g.*, diabetic retinopathy, retinal dystrophy, and retinal degeneration caused by infantile malignant osteopetrosis, ceroid-lipofuscosis, or cholestasis, or caused by photodegeneration, trauma, axotomy, neurotoxic-excitatory degeneration, or ischemic neuronal degeneration.

"Peripheral neuropathy" refers to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be attributed uniquely to an equally wide number of causes. For example, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent. Examples include but are not limited to distal sensorimotor neuropathy, or autonomic neuropathies such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine.

"Heart failure" refers to an abnormality of cardiac function where the heart does not pump blood at the rate needed for the requirements of metabolizing tissues. Heart failure includes a wide range of disease states such as congestive heart failure, myocardial infarction, and tachyarrhythmia.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal herein is human.

As used herein, "ACE inhibitor" refers to angiotensin-converting enzyme inhibiting drugs which prevent the conversion of angiotensin I to angiotensin II. The ACE inhibitors may be beneficial in congestive heart failure by reducing systemic vascular resistance and relieving circulatory congestion. The ACE inhibitors include but are not limited to those designated by the trademarks Accupril® (quinapril), Altace® (ramipril), Capoten® (captopril), Lotensin® (benazepril), Monopril® (fosinopril), Prinivil® (lisinopril), Vasotec® (enalapril), and Zestril® (lisinopril). One example of an ACE inhibitor is that sold under the trademark Capoten®. Generically referred to as captopril, this ACE inhibitor is designated chemically as 1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline.

II. Modes for Practicing the Invention

1. CT-1 Polypeptides

Preferred polypeptides of this invention are substantially homogeneous CT-1 polypeptide(s), having the biological properties of being myocyte hypertrophic and of stimulating the development of chick ciliary neurons in a CNTF assay. More preferred CT-1s are isolated mammalian protein(s) having hypertrophic, anti-arrhythmic, inotropic, and neurological activity. Most preferred polypeptides of this invention are mouse and human CT-1s including fragments thereof having hypertrophic, anti-arrhythmic, inotropic, and neurological activity. Optionally these murine and human CT-1s lack glycosylation. WO 9529237, which published November, 02, 1995, and which is incorproated herein by reference, discloses CT-1 nucleic acid and protein sequences and certain uses of CT-1.

Optional preferred polypeptides of this invention are biologically active CT-1 variant(s) with an amino acid sequence having at least 70% amino acid sequence identity with the murine CT-1 of Fig. 1, preferably at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% (*i.e.*, 70-100%, 75-100%, 80-100%, 85-100%, 90-100%, and 95-100% sequence identity, respectively). Alternatively, the preferred biologically active CT-1 variant(s) have an amino acid sequence having at least 70%, preferably at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% amino acid sequence identity with the human CT-1 sequence of Fig. 5 (i.e., 70-100%, 75-100%, 80-100%, 85-100%, 90-100%, and 95-100% sequence identity, respectively).

The CT-1 cloned from murine embryoid bodies has the following characteristics:

(1) It has a molecular weight of about 21-23 kD as measured by reducing SDS-PAGE;

(2) It shows positive activity in the CNTF chick ciliary neuron assay and in the myocyte hypertrophy and ANP-release hypertrophy assays.

More preferred CT-1 polypeptides are those encoded by genomic DNA or cDNA and having the amino acid sequence of murine CT-1 described in Fig. 1 or the amino acid sequence of human CT-1 described in Fig. 5.

Other preferred naturally occurring biologically active CT-1 polypeptides of this invention include prepro-CT-1, pro-CT-1, pre-CT-1, mature CT-1, and glycosylation variants thereof.

Still other preferred polypeptides of this invention include CT-1 sequence variants and chimeric CT-1s. Ordinarily, preferred CT-1 sequence variants are biologically active CT-1 variants that have an amino acid sequence having at least 70% amino acid sequence identity with the human or murine CT-1, preferably at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%. An exemplary preferred CT-1 variant is a C-terminal domain CT-1 variant in which one or more of the basic or dibasic amino acid residue(s) (*e.g.*, R or K) is substituted with a non-basic amino acid residue(s) (*e.g.*, hydrophobic, neutral, acidic, aromatic, gly, pro and the like).

Another exemplary preferred CT-1 sequence variant is a "domain chimera" that consists of the N-terminal residues substituted with one or more, but not all, of the human CNTF residues approximately aligned as shown in Fig. 2. In this embodiment, the CT-1 chimera would have individual or blocks of residues from the human CNTF sequence added to or substituted into the CT-1 sequence at positions corresponding to the alignment shown in Fig. 2. For example, one or more of those segments of CNTF that are not homologous could be substituted into the corresponding segments of CT-1. It is contemplated that this "CT-1-CNTF domain chimera" will have mixed hypertrophic/anti-arrhythmic/inotropic/neurotrophic biological activity.

Other preferred polypeptides of this invention include CT-1 fragments having a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues, preferably about 10-150 residues, that is identical to the sequence of the CT-1 isolated from murine embryoid bodies or to that of the corresponding human CT-1. Other preferred CT-1 fragments include those produced as a result of chemical or enzymatic hydrolysis or digestion of the purified CT-1.

Another aspect of the invention is a method for purifying CT-1 molecules comprising contacting a CT-1 source containing the CT-1 molecules to be purified with an immobilized receptor or antibody polypeptide, under

conditions whereby the CT-1 molecules to be purified are selectively adsorbed onto the immobilized receptor or antibody polypeptide, washing the immobilized support to remove non-adsorbed material, and eluting the molecules to be purified from the immobilized receptor or antibody polypeptide to which they are adsorbed with an elution buffer. The source containing the CT-1 may be a cell suspension of embryoid bodies.

Alternatively, the source containing the CT-1 is recombinant cell culture where the concentration of CT-1 in either the culture medium or in cell lysates is generally higher than in plasma or other natural sources. In this case the above-described immunoaffinity method, while still useful, is usually not necessary and more traditional protein purification methods known in the art may be applied. Briefly, the preferred purification method to provide substantially homogeneous CT-1 comprises: removing particulate debris by, for example, centrifugation or ultrafiltration; optionally concentrating the protein pool with a commercially available protein concentration filter; and thereafter purifying the CT-1 from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; Toyopearl and MONO-Q or MONO-S chromatography; gel filtration using, for example, Sephadex G-75; chromatography on columns that bind the CT-1, and protein A Sepharose columns to remove contaminants such as IgG. One preferred purification scheme for both native and recombinant CT-1 uses a Butyl Toyopearl column followed by a MONO-Q column and a reverse-phase C4 column as described further below.

In another preferred embodiment, this invention provides an isolated antibody capable of binding to the CT-1. A preferred isolated anti-CT-1 antibody is monoclonal (Kohler et al., *Nature*, 256:495-497 (1975); Campbell, *Laboratory Techniques in Biochemistry and Molecular Biology*, Burdon *et al.*, Eds, Volume 13, Elsevier Science Publishers, Amsterdam (1985); and Huse *et al.*, *Science*, 246:1275-1281 (1989)). Preferred isolated anti-CT-1 antibody is one that binds to CT-1 with an affinity of at least about 10^6 L/mole. More preferably, the antibody binds with an affinity of at least about 10^7 L/mole. Most preferably, the antibody is raised against a CT-1 having one of the above-described effector functions. The isolated antibody capable of binding to the CT-1 may optionally be fused to a second polypeptide and the antibody or fusion thereof may be used to isolate and purify CT-1 from a source as described above for immobilized CT-1 polypeptide. In a further preferred aspect of this embodiment, the invention provides a method for detecting the CT-1 *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the CT-1 and detecting if binding has occurred.

The invention also provides an isolated nucleic acid molecule encoding the CT-1 or fragments thereof, which nucleic acid molecule may be labeled or unlabeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under stringent or moderately stringent conditions with, a nucleic acid molecule having a sequence encoding a CT-1. A preferred CT-1 nucleic acid is RNA or DNA that encodes a biologically active CT-1 sharing at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95%, sequence identity with the murine or human CT-1. More preferred isolated nucleic acid molecules are DNA sequences encoding biologically active CT-1, selected from: (a) DNA based on the coding region of a mammalian CT-1 gene (*e.g.*, DNA comprising the nucleotide sequence provided in Fig. 1 or Fig. 5, or fragments thereof); (b) DNA capable of hybridizing to

a DNA of (a) under at least moderately stringent conditions: and (c) DNA that is degenerate to a DNA defined in (a) or (b) which results from degeneracy of the genetic code. It is contemplated that the novel CT-1s described herein may be members of a family of ligands having suitable sequence identity that their DNA may hybridize with the DNA of Fig. 1 or Fig. 5 (or fragments thereof) under low to moderate stringency conditions. Thus, a further aspect of this invention includes DNA that hybridizes under low to moderate stringency conditions with DNA encoding the CT-1 polypeptides.

Preferably, the nucleic acid molecule is cDNA encoding the CT-1 and further comprises a replicable vector in which the cDNA is operably linked to control sequences recognized by a host transformed with the vector. This aspect further includes host cells transformed with the vector and a method of using the cDNA to effect production of CT-1, comprising expressing the cDNA encoding the CT-1 in a culture of the transformed host cells and recovering the CT-1 from the host cell culture. The CT-1 prepared in this manner is preferably substantially homogeneous murine or human CT-1.

The invention further includes a preferred method for treating a mammal having heart failure, or an arrhythmic, inotropic, or neurological disorder, comprising administering a therapeutically effective amount of a CT-1 to the mammal. Optionally, the CT-1 is administered in combination with an ACE inhibitor, such as captopril, in the case of congestive heart failure, or with another myocardiotrophic, anti-arrhythmic, or inotropic factor in the case of other types of heart failure or cardiac disorder, or with a neurotrophic molecule such as, *e.g.*, IGF-I, CNTF, NGF, NT-3, BDNF, NT-4, NT-5, *etc.* in the case of a neurological disorder.

2. Preparation of Natural-Sequence CT-1 and Variants

Most of the discussion below pertains to production of CT-1 by culturing cells transformed with a vector containing CT-1 nucleic acid and recovering the polypeptide from the cell culture. It is further envisioned that the CT-1 of this invention may be produced by homologous recombination, as provided for in WO 91/06667 published 16 May 1991. Briefly, this method involves transforming primary mammalian cells containing endogenous CT-1 gene (*e.g.*, human cells if the desired CT-1 is human) with a construct (*i.e.*, vector) comprising an amplifiable gene (such as dihydrofolate reductase [DHFR] or others discussed below) and at least one flanking region of a length of at least about 150 bp that is homologous with a DNA sequence at the locus of the coding region of the CT-1 gene to provide amplification of the CT-1 gene. The amplifiable gene must be at a site that does not interfere with expression of the CT-1 gene. The transformation is conducted such that the construct becomes homologously integrated into the genome of the primary cells to define an amplifiable region.

Primary cells comprising the construct are then selected for by means of the amplifiable gene or other marker present in the construct. The presence of the marker gene establishes the presence and integration of the construct into the host genome. No further selection of the primary cells need be made, since selection will be made in the second host. If desired, the occurrence of the homologous recombination event can be determined by employing PCR and either sequencing the resulting amplified DNA sequences or determining the appropriate length of the PCR fragment when DNA from correct homologous integrants is present and expanding only those cells containing such fragments. Also if desired, the selected cells may be amplified at this point by stressing the cells with the appropriate amplifying agent (such as methotrexate if the amplifiable gene is DHFR), so that multiple copies of the target gene are obtained. Preferably, however, the amplification step is not conducted until after the second transformation described below.

After the selection step, DNA portions of the genome, sufficiently large to include the entire amplifiable region, are isolated from the selected primary cells. Secondary mammalian expression host cells are then transformed with these genomic DNA portions and cloned, and clones are selected that contain the amplifiable region. The amplifiable region is then amplified by means of an amplifying agent, if not already amplified in the primary cells. Finally, the secondary expression host cells now comprising multiple copies of the amplifiable region containing CT-1 are grown so as to express the gene and produce the protein.

A. Isolation of DNA Encoding CT-1

The DNA encoding CT-1 may be obtained from any cDNA library prepared from tissue believed to possess the CT-1 mRNA and to express it at a detectable level. The mRNA is suitably prepared, for example, from seven-day differentiated embryoid bodies. The CT-1 gene may also be obtained from a genomic library or by *in vitro* oligonucleotide synthesis as defined above assuming the complete nucleotide or amino acid sequence is known.

Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include, e.g.: monoclonal or polyclonal antibodies that recognize and specifically bind to the CT-1; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of the CT-1 cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*, *supra*.

An alternative means to isolate the gene encoding CT-1 is to use PCR methodology as described in section 14 of Sambrook *et al.*, *supra*. This method requires the use of oligonucleotide probes that will hybridize to the CT-1. Strategies for selection of oligonucleotides are described below.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian differentiated embryoid bodies and placental, cardiac, and brain cell lines. More preferably, human embryoid, placental, cardiac, and brain cDNA libraries are screened with the oligonucleotide probes.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ^{32}P-labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Of particular interest is the CT-1 nucleic acid that encodes a full-length polypeptide. In some preferred embodiments, the nucleic acid sequence includes the native CT-1 signal sequence. Nucleic acid having all the

protein coding sequence is obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, *supra*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

B. Amino Acid Sequence Variants of Native CT-1

Amino acid sequence variants of native CT-1 are prepared by introducing appropriate nucleotide changes into the native CT-1 DNA, or by *in vitro* synthesis of the desired CT-1 polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for murine CT-1 in Figure 1 and for human CT-1 in Figure 5. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are CT-1 variants or polypeptide sequences that are the rat homolog of CT-1. The amino acid changes also may alter post-translational processes of the native CT-1, such as changing the number or position of glycosylation sites.

For the design of amino acid sequence variants of native CT-1, the location of the mutation site and the nature of the mutation will depend on the CT-1 characteristic(s) to be modified. For example, candidate CT-1 antagonists or super agonists will be initially selected by locating sites that are identical or highly conserved among CT-1 and other ligands binding to members of the growth hormone (GH)/cytokine receptor family, especially CNTF and leukemia inhibitory factor (LIF). The sites for mutation can be modified individually or in series, *e.g.*, by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

A useful method for identification of certain residues or regions of the native CT-1 polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham *et al.*, *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis is conducted at the target codon or region and the CT-1 variants produced are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from the Fig. 1 or Fig. 5 sequence, and may represent naturally occurring alleles (which will not require manipulation of the native CT-1 DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the CT-1 characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Contiguous deletions ordinarily are made in even numbers of residues, but single or odd numbers of deletions are within the scope hereof. Deletions may be introduced into regions of low homology among CT-1 and other ligands binding to the GH/cytokine receptor family which share the most sequence identity to the human CT-1 amino acid sequence to modify the activity of CT-1. Deletions from CT-1 in areas of substantial homology with one of the receptor binding sites of other ligands that bind to the GH/cytokine receptor family will be more likely to modify the biological activity of CT-1 more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of CT-1 in the affected domain, *e.g.*, beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (*i.e.*, insertions within the mature CT-1 sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Insertions are preferably made in even numbers of residues, but this is not required. Examples of terminal insertions include mature CT-1 with an N-terminal methionyl residue, an artifact of the direct production of mature CT-1 in recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the mature CT-1 molecule to facilitate the secretion of mature CT-1 from recombinant hosts. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertional variants of the native CT-1 molecule include the fusion to the N- or C-terminus of native CT-1 of immunogenic polypeptides, *e.g.*, bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

A third group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the native CT-1 molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of native CT-1 and sites where the amino acids found in the known analogues are substantially different in terms of side-chain bulk, charge, or hydrophobicity, but where there is also a high degree of sequence identity at the selected site within various animal CT-1 species, or where the amino acids found in known ligands that bind to members of the GH/cytokine receptor family and novel CT-1 are substantially different in terms of side-chain bulk, charge, or hydrophobicity, but where there also is a high degree of sequence identity at the selected site within various animal analogues of such ligands (*e.g.*, among all the animal CNTF molecules). This analysis will highlight residues that may be involved in the differentiation of activity of the cardiac hypertrophic, anti-arrhythmic, inotropic, and neurotrophic factors, and therefore, variations at these sites may affect such activities.

Other sites of interest are those in which particular residues of the CT-1 obtained from various species are identical among all animal species of CT-1 and other ligands binding to GH/cytokine receptor family molecules, this degree of conformation suggesting importance in achieving biological activity common to these enzymes. These sites, especially those falling within a sequence of at least three other identically conserved sites,

are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of the native CT-1 are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in the molecule. These sites are identified by inspection of the encoded amino acid sequence, in the case of trypsin, *e.g.*, for an arginyl or lysinyl residue. When protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment, any methionyl residues other than the starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accord with Table 1) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites.

Any cysteine residues not involved in maintaining the proper conformation of native CT-1 also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Nucleic acid molecules encoding amino acid sequence variants of native CT-1 are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of native CT-1.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of native CT-1 DNA. This technique is well known in the art as described by Adelman *et al.*, *DNA*, 2:183 (1983). Briefly, native CT-1 DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of CT-1. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the native CT-1 DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea *et al.*, *Proc. Natl. Acad. Sci. USA*, 75:5765 (1978).

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera *et al.*, *Meth. Enzymol.*, 153:3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook *et al.*, *supra*.

Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of native CT-1, and the other strand (the original template) encodes the native, unaltered sequence of CT-1. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with ^{32}P to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with *Exo*III nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding mutants of native CT-1 with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide

encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of native CT-1. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 μL. The reaction mixture is overlayed with 35 μL mineral oil. The reaction mixture is denatured for five minutes at 100° C, placed briefly on ice, and then 1 μL *Thermus aquaticus (Taq)* DNA polymerase (5 units/μL, purchased from Perkin-Elmer Cetus) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows:

2 min. 55° C

30 sec. 72° C, then 19 cycles of the following:

30 sec. 94° C

30 sec. 55° C, and

30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50 vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, *Gene*, 34:315 (1985). The starting material is the plasmid (or other vector) comprising the native CT-1 DNA to be mutated. The codon(s) in the native CT-1 DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the native CT-1 DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the CT-1 DNA sequence mutated from native CT-1.

C. Insertion of Nucleic Acid into Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding CT-1 is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the nucleic acid to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

The CT-1s of this invention may be produced not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the CT-1 DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native CT-1 signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, yeast alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Patent No. 5,010,182 issued 23 April 1991), yeast acid phosphatase leader, mouse salivary amylase leader, carboxypeptidase leader, yeast BAR1 leader, Humicola lanuginosa lipase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal

described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native human signal sequence (*i.e.*, the CT-1 presequence that normally directs secretion of native CT-1 from human cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal CT-1s, signal sequences from a ligand binding to another GH/cytokine receptor family member, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature CT-1.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV, or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of CT-1 DNA. However, the recovery of genomic DNA encoding CT-1 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the CT-1 DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.*, 1:327 (1982)), mycophenolic acid (Mulligan *et al.*, *Science*, 209:1422 (1980)), or hygromycin (Sugden *et al.*, *Mol. Cell. Biol.*, 5:410-413 (1985)). The three examples given above employ bacterial genes

under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the CT-1 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes CT-1. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of CT-1 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and

-II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding CT-1. This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding CT-1, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979); Kingsman *et al.*, *Gene*, 7:141 (1979); or Tschemper *et al.*, *Gene*, 10:157 (1980)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 (1977)). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi *et al.*, Curr. Genet., 12: 185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology, 8: 135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin

by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, Bio/Technology, 9: 968-975 (1991).

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the CT-1 nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the CT-1 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to CT-1-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native CT-1 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the CT-1 DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of recombinantly produced CT-1 as compared to the native CT-1 promoter.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.*, Nature, 275: 615 (1978); and Goeddel *et al.*, Nature, 281: 544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 (1980) and EP 36,776), and hybrid promoters such as the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80: 21-25 (1983)). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding CT-1 (Siebenlist *et al.*, Cell, 20: 269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding CT-1.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255: 2073 (1980)) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7: 149 (1968); and Holland, Biochemistry, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phos-

phate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

CT-1 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the CT-1 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, 273:113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, Nature, 297: 598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the CT-1 of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA, 78: 993 (1981)) and 3' (Lusky *et al.*, Mol. Cell Bio., 3: 1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, Cell, 33: 729 (1983)), as well as within the coding sequence itself (Osborne *et al.*, Mol. Cell Bio., 4: 1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the CT-1-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding CT-1.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65: 499 (1980).

(viii) Transient Expression Vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding CT-1. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of native CT-1 that are biologically active CT-1.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of CT-1 in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293: 620-625 (1981); Mantei *et al.*, Nature, 281: 40-46 (1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture production of CT-1 is pRK5 (EP 307,247) or pSVI6B (WO 91/08291 published 13 June 1991). The pRK5 derivative pRK5B (Holmes *et al.*, Science, 253: 1278-1280 (1991)) is particularly suitable herein for such expression.

D. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266.710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred

E. coli cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), *E. coli* DH5α, and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype *tonA Δ*; *E. coli* W3110 strain 9E4, which has the complete genotype *tonA Δ ptr3*; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype *tonA ptr3 phoA ΔE15 Δ(argF-lac)169 ΔdegP ΔompT kan*^{r}; *E. coli* W3110 strain 37D6, which has the complete genotype *tonA ptr3 phoA ΔE15 Δ(argF-lac)169 ΔdegP ΔompT Δrbs7 ilvG kan*^{r}; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant *degP* deletion mutation; and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990. Alternatively, *in vitro* methods of cloning, *e.g.*, PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for CT-1-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer *et al.*, *supra*) such as, *e.g.*, *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, J. Bacteriol., 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg *et al.*, *supra*), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol., 28: 265-278 (1988)); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76: 5259-5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, *e.g.*, *Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112: 284-289 (1983); Tilburn *et al.*, Gene, 26: 205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475-479 (1985)).

Suitable host cells for the production of CT-1 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, *e.g.*, Luckow *et al.*, Bio/Technology, 6: 47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature, 315: 592-594 (1985). A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the CT-1 DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the CT-1 is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the CT-1 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are a monkey kidney CV1 cell line transformed by SV40 (COS-7, ATCC CRL 1651); a human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36: 59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52: 456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen

et al., J. Bact., 130: 946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, *etc.*, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology, 185: 527-537 (1990) and Mansour *et al.*, Nature, 336: 348-352 (1988).

E. Culturing the Host Cells

Prokaryotic cells used to produce the CT-1 polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the CT-1 of this invention may be cultured in a variety of media. Commercially available media such as Ham's F-10 (Sigma), F-12 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium ([D-MEM], Sigma), and D-MEM/F-12 (Gibco BRL) are suitable for culturing the host cells. In addition, any of the media described, for example, in Ham and Wallace, Methods in Enzymology, 58: 44 (1979); Barnes and Sato, Anal. Biochem., 102: 255 (1980); U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469; or 4,560,655; U.S. Patent Re. No. 30,985; WO 90/03430; or WO 87/00195 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, aprotinin, and/or epidermal growth factor [EGF]), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of *in vitro* mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

F. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in

turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native CT-1 polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

G. Purification of CT-1 Polypeptide

CT-1 preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. When CT-1 is produced in a recombinant cell other than one of human origin, the CT-1 is completely free of proteins or polypeptides of human origin. However, it is necessary to purify CT-1 from cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to CT-1. As a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the CT-1 from other impurities by one or more steps selected from immunoaffinity chromatography, ion-exchange column fractionation (*e.g.*, on DEAE or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups), gel filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the CT-1, and ethanol or ammonium sulfate precipitation. A protease inhibitor may be included in any of the foregoing steps to inhibit proteolysis. Examples of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin, chymostatin, and benzamidine.

A preferred purification scheme involves adjusting the culture medium conditioned by cells transfected with the relevant clone to 1.5 M NaCl and applying to a Butyl Toyopearl™ column. The column is washed with Tris[hydroxymethyl]aminomethane hydrochloride (TRIS-HCl), pH 7.5, containing NaCl, and the activity eluted with TRIS-HCl, pH 7.5, containing 10 mM Zwittergent™ 3-10 surfactant. The peak of activity is adjusted to 150 mM NaCl, pH 8.0, and applied to a MONO-Q Fast Flow column. This column is washed with TRIS-HCl, pH 8.0, containing NaCl and octyl glucoside. Activity is found in the flow-through fraction. The active material is then applied to a reverse phase C4 column in 0.1% TFA, 10% acetonitrile, and eluted with a gradient of 0.1%

TFA up to 80%. The activity fractionates at about 15-30 kDa on gel filtration columns. It is expected that a chaotrope such as guanidine-HCl is required for resolution and recovery.

CT-1 variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native CT-1, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a CT-1 fusion with another protein or polypeptide, *e.g.*, a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-CT-1 column can be employed to absorb the CT-1 variant by binding it to at least one remaining immune epitope. A protease inhibitor such as those defined above also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native CT-1 may require modification to account for changes in the character of CT-1 or its variants upon production in recombinant cell culture.

H. Covalent Modifications of CT-1 Polypeptides

Covalent modifications of CT-1 polypeptides are included within the scope of this invention. Both native CT-1 and amino acid sequence variants of native CT-1 may be covalently modified. One type of covalent modification included within the scope of this invention is the preparation of a variant CT-1 fragment. Variant CT-1 fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis or by enzymatic or chemical cleavage of the full-length or variant CT-1 polypeptide. Other types of covalent modifications of the CT-1 or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of the CT-1 or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking CT-1 to a water-insoluble support matrix or surface for use in the method for purifying anti-CT-1 antibodies, and vice-versa. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the CT-1 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native CT-1, and/or adding one or more glycosylation sites that are not present in the native CT-1.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the CT-1 polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked

glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native CT-1 sequence (for O-linked glycosylation sites). For ease, the native CT-1 amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the native CT-1 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under Section 2B.

Another means of increasing the number of carbohydrate moieties on the CT-1 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of any carbohydrate moieties present on the CT-1 polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al.*, Arch. Biochem. Biophys., 259: 52 (1987) and by Edge *et al.*, Anal. Biochem., 118: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, Meth. Enzymol., 138: 350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.*, J. Biol. Chem., 257: 3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of CT-1 comprises linking the CT-1 polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

CT-1 also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., (1980).

CT-1 preparations are also useful in generating antibodies, as standards in assays for CT-1 (*e.g.*, by labeling CT-1 for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant CT-1, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. One can screen for enhanced cardiac hypertrophic, anti-arrhythmic, inotropic, or neurotrophic activity, possession of CT-1

antagonist activity, increased expression levels, oxidative stability, ability to be secreted in elevated yields, and the like. For example, a change in the immunological character of the CT-1 molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its hypertrophic, anti-arrhythmic, inotropic, and neurotrophic activities by comparison to the respective activities observed for native CT-1 in the same assay (using, for example, the hypertrophy and neurotrophic assays described in the examples below.) Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

I. Antagonists of CT-1

Antagonists to CT-1 can be prepared by using the predicted family of receptors for CT-1 (the GH/cytokine receptor family, including the CNTF, LIF, and oncostatin M receptor subfamily, most preferably the LIFRβ or a LIFRβ/gp130 complex). Thus, the receptor can be expression cloned; then a soluble form of the receptor is made by identifying the extracellular domain and excising the transmembrane domain therefrom. The soluble form of the receptor can then be used as an antagonist, or the receptor can be used to screen for small molecules that would antagonize CT-1 activity. Transfected cells expressing recombinant receptor find use in screening molecules both for receptor binding and receptor activation agonism or antagonism.

Alternatively, using the murine sequence shown in Figure 1 or the human sequence shown in Figure 5, variants of native CT-1 are made that act as antagonists. Since the GH/cytokine receptor family is known to have two binding sites on the ligand, the receptor binding sites of CT-1 can be determined by binding studies and one of them eliminated by standard techniques (deletion or radical substitution) so that the molecule acts as an antagonist. For example, as discussed herein, Figure 16 indicate regions that can act as antagonists.

Antagonist activity can be determined by several means, including the hypertrophy assay, the neurotrophic assay, and the other CT-1 assays presented herein.

J. Hypertrophy Assay

A miniatured assay is preferably used to assay for hypertrophic activity. In this assay the medium used allows the cells to survive at a low plating density without serum. By plating directly into this medium, washing steps are eliminated so that fewer cells are removed. The plating density is important: many fewer cells and the survival is reduced; many more cells and the myocytes begin to self-induce hypertrophy.

The steps involved are:

(a) plating 96-well plates with a suspension of myocytes at a cell density of about 7.5×10^4 cells per mL in D-MEM/F-12 medium supplemented with at least insulin, transferrin, and aprotinin;

(b) culturing the cells;

(c) adding a substance to be assayed (such as one suspected of containing a CT-1);

(d) culturing the cells with the substance; and

(e) measuring for hypertrophy.

The medium can be supplemented with additional elements such as EGF that ensure a longer viability of the cells, but such supplements are not essential. D-MEM/F-12 medium is available from Gibco BRL, Gaithersburg, MD, and consists of one of the following media (Table 2):

TABLE 2

Com-ponent	11320 1 x Liquid (mg/L)	11321 1 x Liquid (mg/L)	11330 1 x Liquid (mg/L)	11331 1 x Liquid (mg/L)	12400 Powder (mg/L)	12500 Powder (mg/L)
AMINO ACIDS:						
L-Ala-nine	4.45	4.45	4.45	4.45	4.45	4.45
L-Arg-inine ·HCl	147.50	147.50	147.50	147.50	147.50	147.50
L-Asp-ara-gine ·H_2O	7.50	7.50	7.50	7.50	7.50	7.50
L-Asp-artic acid	6.65	6.65	6.65	6.65	6.65	6.65
L-Cys-teine ·HCl ·H_2O	17.56	17.56	17.56	17.56	17.56	17.56
L-Cys-tine ·2HCl	31.29	31.29	31.29	31.29	31.29	31.29
L-Glu-tamic acid	7.35	7.35	7.35	7.35	7.35	7.35
L-Glu-tamine	365.00	365.00	365.00	365.00	365.00	365.00
Gly-cine	18.75	18.75	18.75	18.75	18.75	18.75
L-His-tidine ·HCl ·H_2O	31.48	31.48	31.48	31.48	31.48	31.48
L-Iso-leu-cine	54.47	54.47	54.47	54.47	54.47	54.47
L-Leu-cine	59.05	59.05	59.05	59.05	59.05	59.05
L-Lys-ine ·HCl	91.25	91.25	91.25	91.25	91.25	91.25
L-Meth-ionine	17.24	17.24	17.24	17.24	17.24	17.24
L-Phen-ylala-nine	35.48	35.48	35.48	35.48	35.48	35.48
L-Pro-line	17.25	17.25	17.25	17.25	17.25	17.25
L-Ser-ine	26.25	26.25	26.25	26.25	26.25	26.25
L-Thre-onine	53.45	53.45	53.45	53.45	53.45	53.45

L-Tryp-tophan	9.02	9.02	9.02	9.02	9.02	9.02
L-Tyro-sine ·2Na ·2H_2O	55.79	55.79	55.79	55.79	55.79	55.79
L-Val-ine	52.85	52.85	52.85	52.85	52.85	52.85
INOR-GANIC SALTS:						
$CaCl_2$ anhyd.	116.60	116.60	116.60	116.60	116.60	116.60
$CuSO_4$ ·5H_2O	0.0013	0.0013	0.0013	0.0013	0.0013	0.0013
Fe $(NO_3)_3$ ·9H_2O	0.05	0.05	0.05	0.05	0.05	0.05
$FeSO_4$ ·7H_2O	0.417	0.417	0.417	0.417	0.417	0.417
KCl	311.80	311.80	311.80	311.80	311.80	311.80
$MgCl_2$	28.64	28.64	28.64	28.64	28.64	28.64
$MgSO_4$	48.84	48.84	48.84	48.84	48.84	48.84
NaCl	6999.50	6999.50	6999.50	6999.50	6999.50	6999.50
$NaHCO_3$	2438.00	2438.00	2438.00	2438.00	--	--
NaH_2PO_4 ·H_2O	62.50	62.50	62.50	--	62.50	62.50
Na_2HPO_4	71.02	71.02	71.02	--	71.02	71.02
$ZnSO_4$ ·7H_2O	0.432	0.432	0.432	0.432	0.432	0.432
OTHER COMPO-NENTS:						
D-Glu-cose	3151.00	3151.00	3151.00	3151.00	3151.00	3151.00
HEPES	--	--	3574.50	3574.50	3574.50	--
Na hypo-xan-thine	2.39	2.39	2.39	2.39	2.39	2.39
Lino-leic acid	0.042	0.042	0.042	0.042	0.042	0.042
Lipoic acid	0.105	0.105	0.105	0.105	0.105	0.105
Phenol red	8.10	8.10	8.10	8.10	8.10	8.10

Pu-tres-cine $\cdot 2H_2O$	0.081	0.081	0.081	0.081	0.081	0.081
Sodium pyru-vate	55.00	55.00	55.00	55.00	55.00	55.00
VITA-MINS:						
Biotin	0.0035	0.0035	0.0035	0.0035	0.0035	0.0035
D-Ca panto-then-ate	2.24	2.24	2.24	2.24	2.24	2.24
Cho-line chlor-ide	8.98	8.98	8.98	8.98	8.98	8.98
Folic acid	2.65	2.65	2.65	2.65	2.65	2.65
i-Ino-sitol	12.60	12.60	12.60	12.60	12.60	12.60
Nia-cin-amide	2.02	2.02	2.02	2.02	2.02	2.02
Pyrid-oxal $\cdot$HCl	2.00	--	2.00	--	2.00	2.00
Pyrid-oxine $\cdot$HCl	0.031	2.031	0.031	2.031	0.031	0.031
Ribo-flavin	0.219	0.219	0.219	0.219	0.219	0.219
Thi-amine $\cdot$HCl	2.17	2.17	2.17	2.17	2.17	2.17
Thy-midine	0.365	0.365	0.365	0.365	0.365	0.365
Vi-tamin B_{12}	0.68	0.68	0.68	0.68	0.68	0.68

The preferred hypertrophy assay comprises:

(a) precoating the wells of 96-well tissue culture plates with a medium containing calf serum, preferably D-MEM/F-12 medium containing 4% fetal calf serum, wherein preferably the wells are incubated with the medium for about eight hours at about 37° C;

(b) removing the medium;

(c) plating a suspension of myocytes in the inner 60 wells at 7.5 x 10^4 cells per mL in D-MEM/F-12 medium supplemented with insulin, transferrin, and aprotinin;

(d) culturing the myocytes for at least 24 hours;

(e) adding the test substance;

(f) culturing the cells with the test substance (preferably for about 24-72 hours, more preferably for about 48 hours); and

(g) measuring for hypertrophy, preferably with crystal violet stain.

Preferably the medium used in step (c) is a serum-free medium also containing penicillin/streptomycin (pen/strep) and glutamine. Most preferably, the medium contains 100 mL D-MEM/F-12, 100 µL transferrin (10 mg/mL), 20 µL insulin (5 mg/mL), 50 µL aprotinin (2 mg/mL), 1 mL pen/strep (JRH Biosciences No. 59602-77P), and 1 mL L-glutamine (200 mM).

The assay capacity of 1000 single samples a week coupled with the small sample size requirement of 100 µL or less has enabled an expression cloning and protein purification that would have been impossible to accomplish using the current methods available.

Another method for assaying hypertrophy involves measuring for atrial natriuretic peptide (ANP) release by means of an assay that determines the competition for binding of ^{125}I-rat ANP for a rat ANP receptor A-IgG fusion protein. The method suitable for use is similar to that used for determining gp120 using a CD4-IgG fusion protein described by Chamow *et al.*, Biochemistry, 29: 9885-9891 (1990).

The basis for the isolation and characterization of the novel hypertrophy factor, CT-1, is the miniaturized high through-put hypertrophy assay system, which was developed in a 96 well format, in which hypertrophy is scored on individual myocardial cells following crystal-violet staining of neonatal rat cardiac myocytes. This assay was used in combinatio with an *in vitro* model of embryonic stem cell cardiogenesis (Miller-Hance *et al.*, *Journal of Biological Chemistry*, 268:25244-25252 (1993)). These totipotent stem cells can differentiate into multi-cellular cystic embryoid bodies (EBs) when cultured in the absence of a fibroblast feeder layer, or without LIF. Since these embryoid bodies spontaneously beat and display cardiac specific markers, it has been suggested that they may serve as a vital source of novel factors that can induce a hypertrophic response *in vitro*)Miller-Hance *et al.*, *Journal of Biological Chemistry*, 268:25244-25252 (1993); Chien, *Science*, 260:916-917 (1993)). By dual immunofluorescence staining of cultured myocardial cells incubated with EB conditioned medium, it was observed that embryoid bodies elaborate a factor that can induce an *in vitro* hypertrophic response in the cultured assay system. This response includes an increase in myocyte size, induction of the expression of ANF, and the assembly of sarcomeric proteins (MLC-2v) into organized contractile units. The hypertrophy assay system was then used to expression clone this factor, which proved to be the novel cytokine, CT-1. These studies document the utility of using expression cloning approaches to identify novel growth factors and cytokines from this *in vitro* model of embryonic stem cell differentiation. This assay system will be of interest in the isolation of other novel cytokines derived from precursors of other differentiated cell types found in EBs, i.e., neurogenic, skeletal myogenic, and hematopoietic precursors.

K. Neurotrophic Assay

The assay used for ciliary ganglion neurotrophic activity described in Leung, Neuron, 8: 1045-1053 (1992) is suitable herein. Briefly, ciliary ganglia are dissected from E7-E8 chick embryos and dissociated in trypsin-EDTA (Gibco 15400-013) diluted ten fold in phosphate-buffered saline for 15 minutes at 37° C. The ganglia are washed free of trypsin with three washes of growth medium (high glucose D-MEM supplemented with 10% fetal bovine serum, 1.5 mM glutamine, 100 µg/mL penicillin, and 100 µg/mL strepomycin), and then gently triturated in 1 mL of growth medium into a single-cell suspension. Neurons are enriched by plating this

cell mixture in 5 mL of growth media onto a 100-mm tissue culture dish for 4 hours at 37° C in a tissue culture incubator. During this time the non-neuronal cells preferentially stick to the dish and neurons can be gently washed free at the end of the incubation. The enriched neurons are then plated into a 96-well plate previously coated with collagen. In each well, 1000 to 2000 cells are plated, in a final volume of 100 to 250 µL, with dilutions of the CT-1 to be tested. Following a 2-4-day incubation at 37° C, the number of live cells is assessed by staining live cells using the vital dye metallothionine (MTT). One-fifth of the volume of 5 mg/mL MTT (Sigma M2128) is added to the wells. After a 2-4-hour incubation at 37° C, live cells (filled with a dense purple precipitate) are counted by phase microscopy at 100X magnification.

3. Uses and Therapeutic Compositions and Administration of CT-1

As disclosed herein, CT-1 activates downstream cellular responses via the heterodimerization of gp130 and LIFRβ. The expression pattern of CT-1 and pleiotropic activities suggest that it may have important functions, not only in the cardiac context, but in extra-cardiac tissues as well. CT-1 acts to maintain normal embryonic growth and morphogenesis, as well as physiological homeostasis in the adult.

CT-1 is believed to find use as a drug for treatment of mammals (*e.g.*, animals or humans) *in vivo* having heart failure, arrhythmic or inotropic disorders, and/or peripheral neuropathies and other neurological disorders involving motor neurons or other neurons in which CNTF is active. CT-1 has additional uses as shown herein.

For example, CT-1 may be useful in treating congestive heart failure in cases where ACE inhibitors cannot be employed or are not as effective. CT-1 optionally is combined with or administered in concert with other agents for treating congestive heart failure, including ACE inhibitors.

The effective amount of ACE inhibitor to be administered, if employed, will be at the physician's or veterinarian's discretion. Dosage administration and adjustment is done to achieve optimal management of congestive heart failure and ideally takes into account use of diuretics or digitalis, and conditions such as hypotension and renal impairment. The dose will additionally depend on such factors as the type of inhibitor used and the specific patient being treated. Typically the amount employed will be the same dose as that used if the ACE inhibitor were to be administered without CT-1.

Thus, for example, a test dose of enalapril is 5 mg, which is then ramped up to 10-20 mg per day, once a day, as the patient tolerates it. As another example, captopril is initially administered orally to human patients in a test dose of 6.25 mg and the dose is then escalated, as the patient tolerates it, to 25 mg twice per day (BID) or three times per day (TID) and may be titrated to 50 mg BID or TID. Tolerance level is estimated by determining whether decrease in blood pressure is accompanied by signs of hypotension. If indicated, the dose may be increased up to 100 mg BID or TID. Captopril is produced for administration as the active ingredient, in combination with hydrochlorothiazide, and as a pH stabilized core having an enteric or delayed release coating which protects captopril until it reaches the colon. Captopril is available for administration in tablet or capsule form. A discussion of the dosage, administration, indications and contraindications associated with captopril and other ACE inhibitors can be found in the *Physicians Desk Reference*, Medical Economics Data Production Co., Montvale, NJ. 2314-2320 (1994).

CT-1 is also potentially useful in the generation, maturation, and survival of oligodendrocytes *in vitro* for protection of oligodendrocytes against natural and tumor necrosis factor-induced death, in the survival and differentiation of astrocytes and the induction of type-2 astrocyte development, and in the stimulation of the

recombinant production of low-affinity nerve growth factor receptor and CD-4 by rat central nervous system (CNS) microglia.

CT-1 is also potentially useful in having a trophic effect on denervated skeletal muscle. In addition, it is expected to have the proliferative responses and binding properties of hematopoietic cells transfected with low-affinity receptors for leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor, to regulate fibrinogen gene expression in hepatocytes by binding to the interleukin-6 receptor, to have trophic actions on murine embryonic carcinoma cells, to be an endogenous pyrogen, and to have a mitogenic effect on human IMR 32 neuroblastoma cells.

In addition, CT-1 is expected to enhance the response to nerve growth factor of cultured rat sympathetic neurons, to maintain motoneurons and their target muscles in developing rats, to induce motor neuron sprouting *in vivo*, to promote the survival of neonatal rat corticospinal neurons *in vitro*, to prevent degeneration of adult rat substantia nigra dopaminergic neurons *in vivo*, to alter the threshold of hippocampal pyramidal neuron sensitivity to excitotoxin damage, to prevent neuronal degeneration and promote low-affinity NGF receptor production in the adult rat CNS, and to enhance neuronal survival in embryonic rat hippocampal cultures.

CT-1 induces a phenotypic switch in sympathetic neurons and it promotes the survival of dopaminergic neurons from the central nervous system and ciliary neurons from the periphery

These activities translate into the treatment of all neurodegenerative diseases by CT-1, including peripheral neuropathies (motor and sensory), ALS, Alzheimer's disease, Parkinson's disease, stroke, Huntington's disease, and ophthalmologic diseases, for example, those involving the retina.

As shown herein CT-1 shares at least some of the growth inhibitory activities of the IL-6 family cytokines. CT-1 has the potential for use as a therapeutic non-proliferative agent for suppressing some forms of myeloid leukaemia as well as a reagent for modifying macrophage function and other responses to infections. CT-1 was 6 fold more potent than LIF in inhibiting the uptake of 3H-thymidine by M1 cells and thus the growth of the myeloid leukemia cell line. CT-1 inhibits the growth of the mouse myeloid leukemia cell line, M1, and induces its differentiation into a macrophage-like phenotype. CT-1 does not mimic the activity of IL-6 in promoting B cell expansion. Unlike IL-6, CT-1 has the advantage of not stimulating the growth of several B cell lymphomas, myelomas, and plasmacytomas. Thus, CT-1 will find use in treating lymphomas and leukemias, preferably B-cell and myeloid leukemias and patients with certain infections. Since CT-1 is useful the treatment of patients with some forms of myeloid leukaemia and patients with certain infections, the present invention also extends to pharmaceutical compositions comprising CT-1, particularly human CT-1, either completely or in part, produced for example using cloned CT-1-encoding DNA sequences or by chemical synthesis, and to pharmaceutical compositions of analogues of CT-1, for example produced by chemical synthesis or derived by mutagenesis of aforesaid cloned CT-1-encoding DNA sequences. The pharmaceutical compositions may also contain at least one other biological regulator of blood cells, such as G-CSF or GM-CSF. Furthermore, the invention also extends to diagnostic reagents for use in detecting genetic rearrangements, alterations or lesions associated with the human CT-1 gene in diseases of blood cell formation, including leukaemia and congenital diseases associated with susceptibility to infection. CT-1 can be used in the treatment of a wide variety of neoplastic conditions, such as carcinomas, sarcomas, melanomas, lymphomas, leukemias, which may affect a wide variety of organs, including the blood, lungs, mammary organ, prostate, intestine, liver, heart, skin,

pancreas, and brain. CT-1 can be used *in vitro* to eliminate malignant cells from marrow for autologous marrow transplants or to inhibit proliferation or eliminate malignant cells in other tissue, e.g. blood, prior to reinfusion.

CT-1 can also be used as a treatment in disorders of the hematopoietic system, especially as a means of stimulating hematopoiesis in patients with suppressed bone marrow function, for example, patients suffering from aplastic anemia, inherited or acquired immune deficiency, or patients undergoing radiotherapy or chemotherapy.

Antagonists to CT-1 can also be used for treating a wide variety of wounds including substantially all cutaneous wounds, corneal wounds, and injuries to the epithelial-lined hollow organs of the body and those involving myocytes and neurons. Wounds suitable for treatment include those resulting from trauma such as burns, abrasions, cuts, and the like as well as from surgical procedures such as surgical incisions and skin grafting. Other conditions suitable for treatment with the CT-1 antagonists include chronic conditions, such as chronic ulcers, diabetic ulcers, and other non-healing (trophic) conditions. Preferably, a CT-1 antagonist is incorporated in physiologically-acceptable carriers for local or site-specific application to the affected area. The nature of the carriers may vary widely and will depend on the intended location of application. If desired, it will be possible to incorporate CT-1 antagonist compositions in bandages and other wound dressings to provide for continuous exposure of the wound to the peptide. Aerosol applications also find use. The antagonist will be present in an amount effective to suppress CT-1 inhibition of epithelial cell proliferation. The compositions will be applied topically to the affected area, typically as eye drops to the eye or as creams, ointments or lotions to the skin. In the case of eyes, frequent treatment is desirable, usually being applied at intervals of 4 hours or less. On the skin, it is desirable to continually maintain the treatment composition on the affected area during healing, with applications of the treatment composition from two to four times a day or more frequently.

CT-1 maintains the undifferentiated phenotype of embryonic stem cells. CT-1 can promote cell survival and acts as an anti-apoptotic factor during mouse embryogenesis. Thus CT-1 will find use in techniques in which undifferentiated ES cells are useful as well as techniques in which control of their differentiation is useful For example, CT-1 will find use to maintain the undifferentiated state of embryonic stem cells during recombinant DNA transformation and their synchronized differentiation in methods such as gene cloning and creating transgenic animals. CT-1 also find use in artificial insemination techniques. Thus, in one preferred embodiment, CT-1 is used in the enhancement of development and maintenance of animal or mammalian embryos and to enhance impregnation.

A major difficulty associated with present in vitro fertilization (IVF) and embryo transfer (ET) programs, particularly in humans, is the success rate "achieved" on implantation of fertilized embryos. Currently, in human IVF programs, the implantation rate may be as low as 10%, leading to the present practice of using up to four fertilized embryos in each treatment which, in turn, leads occasionally to multiple births. Accordingly, there is a need to improve the implantation rate in human IVF programs. Similarly, in IVF and ET treatments in domestic animals such as sheep, cattle, pigs and goats, it is highly desirable for economic reasons to have as high an implantation rate as possible so as to reduce the numbers of fertilized embryos lost and unsuccessful treatment procedures performed. Furthermore, as with human IVF procedures, the practice of transferring more than one embryo to the recipient animal to ensure pregnancy can result in unwanted multiple births. One major constraint with embryo transfer is the need to hold embryos in culture media for either relatively short periods

of time, perhaps only a few hours prior to transfer or for longer periods of some days, after micromanipulation. In the development of a mammalian embryo, the fertilized egg passes through a number of stages including the morula and the blastocyst stages. In the blastocyst stage, the cells form an outer cell layer known as the trophectoderm (which is the precursor of the placenta) as well as an inner cell mass (from which the whole of the embryo proper is derived). The blastocyst is surrounded by the zona pellucida, which is subsequently lost when the blastocyst "hatches". The cells of the trophectoderm are then able to come into close contact with the wall of the uterus in the implantation stage. Prior to formation of the embryo proper by the inner cell mass by gastrulation, the whole cell mass may be referred to as "pre-embryo." Embryo mortality has been attributed to incomplete hatching of the blastocyst from the zona pellucida and/or unsuccessful implantation of the embryo to the uterine wall, possibly due to spontaneous differentiation of the embryonic stem cells (ES) during their period in culture prior to transplantation. CT-1 can be included in an *in vitro* embryo culture medium to enhance the hatching process leading to an increased number of embryos completing the development stage by undergoing developmental changes associated with implantation. Thus, CT-1 is an embryo protective agent. As a result, the implantation rates for IVF and ET programs can be significantly improved by the use of CT-1 in the in vitro embryo culture medium. Furthermore, media containing CT-1 is suitable for use in early manipulative procedures on the oocyte/embryo such as in vitro fertilization, embryo splitting and nuclear transfer where survival rates of embryos are low. CT-1 also has important applications in the growth of totipotent stem cell lines for cloning for inclusion into the media used for the transport of cooled or frozen embryos/semen. Thus a method for enhancing the impregnation rate in an animal with one or more embryos is provided which comprises the steps of maintaining and/or developing the embryos in a medium containing an effective amount of CT-1 for sufficient time and under appropriate conditions and then implanting the embryos into the animal. By "impregnation" means the rate of successful implantations and subsequent development of a fertilized embryo. Also provided is a method for maintaining embryos or pre-embryos in culture while retaining viability for use in embryo transfer and/or genetic manipulation which method includes culturing the embryos in a medium containing an effective amount of CT-1 for sufficient time and under appropriate conditions. This method of maintaining the viability of embryos in culture has potential for allowing genetic manipulation of the whole embryo. Such successful genetic manipulation is restricted at the present time due to the limited amount of time available to perform experiments on viable embryos. The method also may be advantageous in maintaining viability of embryos under transport conditions and may also be beneficial in the storage of embryos when compared to techniques currently employed. Another aspect of the present invention relates to a method for enhancing the in vitro development of a mammalian embryo to the implantation stage, which method comprises the step of culturing the embryo in vitro in a culture medium containing an effective amount of mammalian CT-1. As is demonstrated below the inclusion of CT-1 in the culture medium prior to the formation of the blastocyst, or both prior to and following blastocyst formation, also increases the number of pre-embryos completing the developmental stage by undergoing development changes associated with implantation. The addition of CT-1 also reduces the number of pre-embryos degenerating while in culture. As a result, the implantation rate for IVF and ET programs can be significantly improved by use of CT-1 in the in vitro culture medium. The present invention, also extends to a method for *in vitro* fertilization and subsequent implantation of a mammalian embryo which is characterized in that the embryo is cultured in vitro in a culture medium containing an effective amount

of mammalian CT-1 prior to transfer into animal or mammalian host, where "host" is defined as a suitably receptive female animal or mammal. A further aspect of the present invention relates to a non-human animal and in particular a chimeric non-human animal or transgenic progeny of said animal generated by known techniques using ES cells which have been maintained in vitro in CT-1-containing culture medium. In accordance with this aspect of the present invention, ES cells are derived from animal embryos passaged in a culture medium containing CT-1 wherein said ES cells have additional genetic material inserted therein. The transgenic animals contemplated include nonhuman mammals such as livestock and ruminant animals and domestic animals. The present invention is also directed to composition comprising an effective amount of CT-1 in combination with an animal (e.g. mammalian) embryo maintaining medium. The present invention also provides a composition having embryotrophic and/or embryo protective properties comprising CT-1. The amount of CT-1 used in accordance with the present invention is that required to maintain and/or develop embryos and/or enhance impregnation. Generally it is in the range of 0.1 ng/ml to 10,000 ng/ml, preferably 1 ng/ml to 1000 ng/ml.

CT-1 also finds use to produce a mammalian pluripotential embryonic stem cell composition which can be maintained on feeder layers and give rise to embryoid bodies and multiple differentiated cell phenotypes in monolayer culture. Provided is a method of making a pluripotential embryonic stem cell by administering a growth enhancing amount of basic fibroblast growth factor, CT-1, membrane associated steel factor, and soluble steel factor to primordial germ cells under cell growth conditions, thereby making a pluripotential embryonic stem cell. A "pluripotential embryonic stem cell" as used herein means a cell which can give rise to many differentiated cell types in an embryo or adult, including the germ cells (sperm and eggs). This cell type is also referred to as an "ES cell." Only those mammals which can be induced to form ES cells by the described methods are within the scope of the invention. Although not a requirement for application of this embodiment of the invention, the ES cells may be capable of indefinite maintenance, typically at least 15 days. Once the ES cells are established, they can be genetically manipulated to produce a desired characteristic. For example, the ES cells can be mutated to render a gene non-functional, e.g. the gene associated with cystic fibrosis or an oncogene. Alternatively, functional genes can be inserted to allow for the production of that gene product in an animal, e.g. growth hormones or valuable proteins. The invention also provides a composition comprising pluripotential ES cells and/or primordial germ cells and/or embryonic ectoderm cells and CT-1, an FGF, membrane associated SF, and soluble SF wherein the factors are present in amounts to enhance the growth of and allow the continued proliferation of the cell. Growth and proliferation enhancing amounts can vary. Generally, 0.5 to 500 ng factor/ml of culture solution is adequate. Preferably, the amount is between 10 to 20 ng/ml. Alternatively, CT-1 can be used to maintain ES cells. In this case, the amounts of CT-1, FGF, and SF necessary to maintain ES cells can be much less than that required to enhance growth or proliferation to become ES cells. In addition, CT-1, FGF, or SF may not be required for maintenance of ES cells. The invention also provides a method of making a pluripotential ES cell comprising administering a growth enhancing amount of CT-1, basic FGF, membrane associated SF, and soluble SF to primordial germ cells and/or embryonic ectoderm cells under cell growth conditions, thereby making a pluripotential ES cell. This method can be practiced utilizing any animal cell, especially mammal cells including mice, rats, rabbits, guinea pigs, goats, cows, pigs, humans, etc. The ES cell produced by this method is also contemplated. "Cell growth conditions" are set forth in the Examples. However, many alterations to these conditions can be made and are routine in the art. Since

the invention provides ES cells generated for virtually any animal, the invention provides a method of using the ES cells to contribute to chimeras in vivo comprising injecting the cell into a blastocyst and growing the blastocyst in a foster mother. Alternatively, aggregating the cell with a morula stage embryo and growing the embryo in a foster mother can be used to produce a chimera. The ES cells can be manipulated to produce a desired effect in the chimeric animal. Alternatively, the ES cells can be used to derive cells for therapy to treat an abnormal condition. For example, derivatives of human ES cells could be placed in the brain to treat a neurodegenerative disease.

CT-1 will stimulate the proliferation of satellite cells and the subsequent development of myoblasts. Accordingly, provided are methods of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts which includes the steps of contacting said cells with a stimulation-effective amount of CT-1 for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts. The stimulation-effective amount of CT-1 can be administered simultaneously or in sequential combination with one or more other cytokines, for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts. Also provided are methods of myoblast transfer therapy which include the steps of contacting mammalian satellite cells with a proliferation- and/or differentiation-effective amount of CT-1 for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts and then administering said myoblasts at multiple sites into muscles. In an alternative to this embodiment, CT-1 is used in simultaneous or sequential combination with one or more other cytokines. Accordingly, a cell activating composition comprising CT-1 in combination with one or more other cytokines, and one or more physiologically acceptable carriers and/or diluents is provided. And there is provided a pharmaceutical composition for stimulating the proliferation and/or differentiation of satellite cells which includes CT-1 and one or more other cytokines and one or more pharmaceutically acceptable carriers and/or diluents. In one preferred embodiment, the cytokines in optional combination with CT-1 include IL-6 and/or TGF alpha and/or FGF. The methods and compositions find use especially in relation to primary, genetically determined, muscle myopathies, the most severe and the most common of which is Duchenne muscular dystrophy (DMD). Because of the size and complexity of the DMD gene, it is unlikely that genetic manipulation will be possible in the near future. However, an effective approach involves the growing of myoblasts in culture derived from normal mammals and injecting them, at multiple sites, into muscles of the patient to result in the muscles containing dystrophin whereas previously there was little or none. Thus human myoblasts, grown in culture, are injected at multiple sites into muscles of DMD. This approach is applicable to all primary myopathies, not only DMD. At present, techniques of culturing myoblasts utilize medium to long term culture with varying concentrations of the expensive reagent fetal calf serum. Thus, accelerating myoblast differentiation and growth should be significant advance toward reducing the cost of myoblast production and facilitate therapy. CT-1 alone, or in combination with other cytokines such as IL-6 and/or TGF alpha and/or FGF, will provide this acceleration. Accordingly, provided herein is a method (and compositions for same) of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts which includes the steps of contacting said satellite cells with a stimulation-effective amount of CT-1, alone or in combination with other cytokines such as IL-6 and/or TGF alpha and/or FGF, for a time and under conditions sufficient to stimulate the satellite cells. In these methods the satellite cells are most preferably from the same mammal to be treated, less preferably from the same species

of mammal, and least preferably from different mammals. The mammal can be human, mouse, a livestock or a pet animal. Most preferably CT-1 and the satellite cells are from the same species of mammal. CT-1 can be provided at a concentration of from about 0.1 to about 1000 ng/ml, and more preferably from a concentration of from about 1 to 100 ng/ml.

CT-1 can be involved in the repair of injured muscle and the maintenance of cellular homeostasis. For example, the prominent expression of CT-1 in skeletal muscle indicates that CT-1 will serve to promote the survival of skeletal muscle cells during periods of muscle injury. This is consistent with the finding that in skeletal muscle, LIF and CNTF were found to be involved in the repair of injured muscle (Barnard *et al., J Neurol Sci.*, 123:108-113 (1994); Helgren *et al., Cell*, 76:493-504 (1994)). This function of CT-1 is consistent with the enlarging role of the gp130 dependent cytokines in promoting cell survival. In addition, the level of CT-1 expression in the mature heart and other tissues is consistent with its supportive role to maintain tissue survival in these tissues. In this regard, previous studies have demonstrated that LIF and CNTF can promote neuronal cell survival *in vitro* (Oppenheim *et al., Science*, 251:1616-1618 (1991); Martinou *et al., Neuron*, 8:737-744 (1992)). In addition, analysis of LIF deficient mice suggests that LIF may be required for the microenvironment to maintain the survival of hematopoietic cells (Escary *et al., Nature*, 363:361-364 (1993)). Although the members of the IL-6 family share a great degree of functional redundancy, individual family members may have their own specific target tissues and divergent functions, based upon the localized distribution and density of the cytokines and their receptors. CT-1 can block viral induced apoptosis of neonatal cardiac muscle cells following infection with cardiomyopathic viruses.

As shown herein, CT-1 is a multi-functional cytokine which shares several biological activities with other members of the IL-6 cytokine family. CT-1 and LIF have similar activities in the *in vitro* assay systems examined thus far. Accordingly, CT-1 is expected to find use in the medical treatment uses known for LIF. Figure 21 is a schematic that summarizes the diverse bioactivities of CT-1 in a wide variety of cell types. These activities include the ability of CT-1 to inhibit embryonic stem cell differentiation and aortic endothelial cell proliferation, thus CT-1 will function in regulating development. Like other IL-6 family members, CT-1 induces acute phase proteins in hepatocytes and thus will modulate local inflammatory processes, and play a role as an acute phase mediator *in vivo* (see also Peters *et al., FEBS Letters*, 372:177-180 (1995)). CT-1 or its antagonists will be useful in the treatment of arthritis and inflammatory pathologies. During the inflammatory reaction, substantial modifications occur in the synthesis of a group of plasma proteins called acute-phase proteins. Some of these proteins-including fibrinogen, reactive protein C, haptoglobin are increased during the acute-phase reaction, whereas others such as albumin and transferrin are reduced. The alteration of these proteins, in particular fibrinogen, is responsible for the modifications in the plasma viscosity and for the increase in the speed of sedimentation which are observed in the inflammation. Because of their correlation with clinical parameters during the development and the therapeutic remissions observed in rheumatoid arthritis, some of these acute-phase proteins have been used as a criterion for evaluating the disease (Mallya *et al., J. Rheumatol.* 9:224-8 (1982); Thompson *et al., Arthritis Rheum.* 30:618-23 (1987)). Accordingly, a method is provided for treating a mammal afflicted with arthritis or an inflammatory disease, including those related to autoimmune diseases. The method includes the step of administering to the mammal an amount of compound which is effective for alleviation of the condition. Inflammatory states in mammals include, but are not limited to, allergic and

asthmatic manifestations, dermatological diseases, inflammatory diseases, collagen diseases, reperfusion injury and stroke, infections, and lupus erythematosus. Treatment of both acute and chronic diseases are possible. Preferred diseases for treatment are arthritis, asthma, allergic rhinitis, inflammatory bowel disease (IBD), psoriasis, reperfusion injury and stroke. Other disorders involving acute phase proteins are acute lymphoblastic leukemia (ALL), acute graft versus host disease (aGvHD), chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL), type 1 diabetes, aplastic anemia (AA), Crohn's Disease, and scleroderma. Additional inflammatory conditions include patients with severe burns, kidney transplants, acute infections of the central nervous system and septic shock.

Furthermore, CT-1 like LIF, inhibits the proliferation and induces the differentiation of a mouse myeloid leukemia cell line. Similar to the activity seen for LIF and CNTF, CT-1 has neuronal function, in that it promotes the survival of cultured dopaminergic neurons and ciliary ganglion neurons and induces a switch in the transmitter phenotype of sympathetic neurons. Thus, while CT-1 was initially isolated on the basis of its actions on cardiac muscle cells, it may also have pleiotropic functions in other organ systems that overlap to a significant extent with the activities other IL-6 family cytokines, preferably those of LIF and OSM, and more preferably those of LIF.

As shown herein, CT-1 signals through and induces tyrosine phosphorylation of the gp130/LIFRβ-heterodimer in cardiac myocytes and other cell types. This does not exclude the possibility that CT-1 may use an alternative signaling pathway via an additional private receptor in some cell types. Members of the IL-6 cytokine family including, IL-11, LIF, CNTF, and OSM trigger downstream signaling pathways in multiple cell types through the homodimerization of gp130 or through the heterodimerization of gp130 and a related transmembrane signal transducer, the LIF receptor subunit LIFRβ (Figure 15B; Gearing *et al.*, *Science*, 255:1434-1437 (1992); Ip *et al.*, *Cell*, 69:1121-1132 (1992); Murakami *et al.*, *Science*, 260:1808-1810 (1993); Davis *et al.*, *Science*, 260:1805-1808 (1993). An anti-gp130 monoclonal antibody was used to determine its effect on CT-1 binding to M1 cells. This neutralizing antibody inhibited CT-1 binding to M1 cells indicating that gp130 is a component of the CT-1 receptor complex. CT-1 and LIF also cross-compete for binding to rat cardiac myocytes and mouse M1 cells indicating that these two ligands act on these cells via the LIF receptor. In addition, c-fos induction by CT-1 and LIF in cardiac myocytes was antagonized by the anti-gp130 monoclonal antibody as well as by a mutated human LIF protein, acting as a LIFRβ-antagonist. A direct demonstration that CT-1 interacts with LIFRβ and gp130 has been shown by the binding of CT-1 to purified soluble gp130 and LIFRβ. Accordingly, CT-1 will find use in disorders, diseases or condition relating to cells expressing the LIFRβ and to its signaling pathways.

As demonstrated by immunoprecipitation with a polyclonal anti-gp130 cytoplasmic peptide antibody and subsequent anti-phosphotyrosine immunoblotting, stimulation of cardiomyocytes with CT-1, LIF, and a combination of IL-6 and soluble IL-6 receptor (sIL-6R) resulted in the rapid tyrosine phosphorylation of gp130. These data indicate that tyrosine phosphorylation of the receptor component gp130 is an early step in CT-1 signaling, as has previously been shown for the other members of the IL-6 cytokine family (Ip *et al.*, *Cell*, 69:1121-1132 (1992); Yin *et al.*, *Journal of Immunology*, 151:2555-2561 (1993); Taga *et al.*, *Proc. Natl Acad. Sci. USA*, 89:10998-11001 (1992)). As determined by immunoblotting with an anti-phosphotyrosine antibody, LIF induced the tyrosine phosphorylation of an additional ~200 kDa protein, which was not

phosphorylated upon stimulation with the IL-6/sIL-6R complex. Based on previous results, this protein most likely corresponds to the LIF receptor subunit LIFRβ (Ip *et al., Cell*, 69:1121-1132 (1992); Davis *et al., Science*, 260:1805-1808 (1993); Boulton *et al., Journal of Biological Chemistry*, 269:11648-11655 (1994)). As shown herein, stimulation of cardiac cells with CT-1 also resulted in the tyrosine phosphorylation of a protein, indistinguishable in size from the LIFRβ. And an LIFRβ antagonist blocked the action of CT-1 in cardiomyocytes. Accordingly, CT-1, like LIF, induces the tyrosine phosphorylation of LIFRβ.

Since CT-1 and LIF appear to have functional redundancy in these assay systems, the possibility exists that CT-1 compensated for the complete loss of LIF during embryonic development and adulthood in these LIF deficient embryos. Alternatively, since LIF is not expressed at very high levels in the embryo, CT-1 may be the endogenous ligand which normally performs this function during mouse embryonic development. If the latter is the case, one might expect severe embryonic defects in CT-1 deficient embryos, analogous to either the LIFRβ deficient or gp130 deficient phenotypes. CT-1 will be involved in the maintenance of normal cardiac growth, morphogenesis, and hypertrophy, which can be analyzed in the basal state and in response to the imposition of a mechanical stimulus for hypertrophy via miniaturized physiological technology (Rockman *et al., Proc. Natl Acad. Sci. USA*, 88:8277-8281 (1991)). This system will allow screening and identification of CT-1 agonists and antagonists. Interestingly, a large disparity between the phenotypes seen in mice lacking the CNTF receptor and mice lacking CNTF have been reported (DeChiara *et al., Cell*, 83:313-322 (1995)). While animals which completely lack the CNTF receptor display prominent motor neuron deficits at birth, mice that lack CNTF appear to be relatively unaffected (DeChiara *et al., Cell*, 83:313-322 (1995)), and do not display any notable abnormalities in the developing nervous system. In addition, LIFRβ deficient neonates also display similar profound motor neuron deficits (Li *et al, Nature*, 378:724-727 (1995)). These studies strongly suggest the possibility that there may be an alternative ligand to CNTF that binds to the CNTF receptor and LIFRβ that is required to maintain normal nervous system development. While the CNTF receptor is not required for CT-1 binding to the gp130/ LIFRβ complex and interaction of CT-1 with the CNTF receptor has not been demonstrated, CT-1 may be this alternative ligand.

CT-1 may also be useful as an adjunct treatment of neurological disorders together with such neurotrophic factors as, *e.g.*, CNTF, NGF, BDNF, NT-3, NT-4, and NT-5.

The nucleic acid encoding the CT-1 may be used as a diagnostic for tissue-specific typing. For example, such procedures as *in situ* hybridization, northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding CT-1 is present in the cell type(s) being evaluated.

Isolated CT-1 polypeptide may also be used in quantitative diagnostic assays as a standard or control against which samples containing unknown quantities of CT-1 may be prepared.

Therapeutic formulations of CT-1 for treating heart failure, neurological disorders, and other disorders are prepared for storage by mixing CT-1 having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, *supra)*, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as

polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics, or polyethylene glycol (PEG).

CT-1 to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. CT-1 ordinarily will be stored in lyophilized form or in solution.

Therapeutic CT-1 compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of CT-1 or CT-1 antibody administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained-release systems as noted below. CT-1 is administered continuously by infusion or by bolus injection. CT-1 antibody is administered in the same fashion, or by administration into the blood stream or lymph. Most preferably, CT-1 or its antagonist is administered locally or site-specifically to better obtain a local or site-specific effect. Such suitable delivery methods are known in the art including implants, pumps, patches, direct injection, and transmucosal delivery. Site-specific delivery can be obtained by gene delivery vectors and viruses and by transplantation of cells expressing CT-1 or an antagonist.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res., 15: 167-277 (1981) and Langer, Chem. Tech., 12: 98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919. EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22: 547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release CT-1 compositions also include liposomally entrapped CT-1. Liposomes containing CT-1 are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar

type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal CT-1 therapy.

An effective amount of CT-1 to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 μg/kg to up to 100 mg/kg of patient body weight or more per day, depending on the factors mentioned above, preferably about 10 μg/kg/day to 10 mg/kg/day. Typically, the clinician will administer CT-1 until a dosage is reached that achieves the desired effect for treatment of the heart, neural, or other dysfunction. For example, the amount would be one which increases ventricular contractility and decreases peripheral vascular resistance or ameliorates or treats conditions of similar importance in congestive heart failure patients. The progress of these therapies is easily monitored by conventional assays.

4. CT-1 Antibody Preparation

(i) Starting Materials and Methods

Immunoglobulins (Ig) and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent No. 4,745,055; EP 256,654; EP 120.694; EP 125,023; EP 255,694; EP 266,663; WO 88/03559; Faulkner *et al.*, Nature, 298: 286 (1982); Morrison, J. Immun., 123: 793 (1979); Koehler *et al.*, Proc. Natl. Acad. Sci. USA, 77: 2197 (1980); Raso *et al.*, Cancer Res., 41: 2073 (1981); Morrison *et al.*, Ann. Rev. Immunol., 2: 239 (1984); Morrison, Science, 229: 1202 (1985); and Morrison *et al.*, Proc. Natl. Acad. Sci. USA; 81: 6851 (1984). Reassorted immunoglobulin chains are also known. See, for example, U.S. Patent No. 4,444,878; WO 88/03565; and EP 68,763 and references cited therein. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA, IgE, IgD, or IgM, but preferably from IgG-1 or IgG-3.

(ii) Polyclonal antibodies

Polyclonal antibodies to CT-1 polypeptides or CT-1 fragments are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of CT-1 or CT-1 fragment and an adjuvant. It may be useful to conjugate CT-1 or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the CT-1 polypeptide or CT-1 fragment, immunogenic conjugates, or derivatives by combining 1 mg or 1 μg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for CT-1 or CT-1 fragment antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same CT-1 or CT-1 fragment, but conjugated to a different protein

and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the CT-1 monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler and Milstein, Nature, 256: 495 (1975), or may be made by recombinant DNA methods (Cabilly *et al.*, *supra*).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the CSF or CSF fragment used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA.

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against CT-1. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107: 220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, Curr. Opinion in Immunol., 5: 256-262 (1993) and Plückthun, Immunol. Revs., 130: 151-188 (1992).

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (Morrison, *et al.*, Proc. Nat. Acad. Sci., 81: 6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-CT-1 monoclonal antibody herein.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a CT-1 and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, *e.g.*, ^{125}I, ^{32}P, ^{14}C, or ^{3}H; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter *et al.*, Nature, 144: 945 (1962); David *et al.*, Biochemistry, 13: 1014 (1974); Pain *et al.*, J. Immunol. Meth., 40: 219 (1981); and Nygren, J. Histochem. and Cytochem., 30: 407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be a CT-1 or an immunologically reactive portion thereof) to compete with the test sample analyte (CT-1) for binding with a limited amount of antibody. The amount of CT-1 in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes

bound. the antibodies generally are insolubilized before or after the competition. so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein (CT-1) to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. David and Greene, U.S. Patent No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme (*e.g.*, horseradish peroxidase).

(iv) Humanized antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, Nature 321, 522-525 (1986); Riechmann *et al.*, Nature 332, 323-327 (1988); Verhoeyen *et al.*, Science 239, 1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al.*, supra), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, J. Immunol., 151: 2296 (1993); Chothia and Lesk, J. Mol. Biol., 196: 901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, Proc. Natl. Acad. Sci. USA, 89: 4285 (1992); Presta *et al.*, J. Immnol., 151: 2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the

residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

(v) Human antibodies

Human monoclonal antibodies can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor, J. Immunol. 133, 3001 (1984); Brodeur, *et al.*, Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, J. Immunol., 147: 86-95 (1991).

It is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*, Proc. Natl. Acad. Sci. USA, 90: 2551 (1993); Jakobovits *et al.*, Nature, 362: 255-258 (1993); Bruggermann *et al.*, Year in Immuno., 7: 33 (1993).

Alternatively, the phage display technology (McCafferty *et al.*, Nature, 348: 552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimicks some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology, 3: 564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, Nature, 352: 624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, J. Mol. Biol., 222: 581-597 (1991), or Griffith *et al.*, EMBO J., 12: 725-734 (1993).

In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks *et al.*, Bio/Technol., 10: 779-783 (1992)). In this method, the affinity of "primary" human antibodies obtained by

phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by Waterhouse *et al.*, Nucl. Acids Res., 21: 2265-2266 (1993).

Gene shuffling can also be used to derive human antibodies from rodent antibodies, where the human antibody has similar affinities and specificities to the starting rodent antibody. According to this method, which is also referred to as "epitope imprinting", the heavy or light chain V domain gene of rodent antibodies obtained by phage display technique is replaced with a repertoire of human V domain genes, creating rodent-human chimeras. Selection on antigen results in isolation of human variable capable of restoring a functional antigen-binding site, i.e. the epitope governs (imprints) the choice of partner. When the process is repeated in order to replace the remaining rodent V domain, a human antibody is obtained (see PCT WO 93/06213, published 1 April 1993). Unlike traditional humanization of rodent antibodies by CDR grafting, this technique provides completely human antibodies, which have no framework or CDR residues of rodent origin.

(vi) Bispecific antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a CT-1, the other one is for any other antigen, and preferably for another ligand that binds to a GH/cytokine receptor family member. For example, bispecific antibodies specifically binding a CT-1 and neurotrophic factor, or two different types of CT-1 polypeptides are within the scope of the present invention.

Methods for making bispecific antibodies are known in the art.

Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305: 537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published 13 May 1993, and in Traunecker *et al.*, EMBO J., 10: 3655-3659 (1991).

According to a different and more preferred approach, antibody-variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant-domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1), containing the site necessary for light-chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the production of at

least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. For further details of generating bispecific antibodies, see, for example, Suresh *et al.*, Methods in Enzymology, 121: 210 (1986).

(vii) Heteroconjugate antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360; WO 92/00373; and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

5. Uses of CT-1 Antibodies

CT-1 antibodies are useful in diagnostic assays for CT-1, *e.g.*, its production in specific cells, tissues, or serum. The antibodies are labeled in the same fashion as CT-1 described above and/or are immobilized on an insoluble matrix. In one embodiment of a receptor-binding assay, an antibody composition that binds to all or a selected plurality of CT-1s is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition to adsorb all CT-1s, and then the immobilized CT-1s are contacted with a plurality of antibodies specific for each CT-1, each of the antibodies being individually identifiable as specific for a predetermined CT-1, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each CT-1 can be determined.

The antibodies of this invention are also useful in passively immunizing patients.

CT-1 antibodies also are useful for the affinity purification of CT-1 from recombinant cell culture or natural sources. CT-1 antibodies that do not detectably cross-react with the rat CT-1 can purify CT-1 free from such protein.

Suitable diagnostic assays for CT-1 and its antibodies are well known *per se*. In addition to the bioassays described in the examples below wherein the candidate CT-1 is tested to see if it has hypertrophic, anti-arrhythmic, inotropic, or neurotrophic activity, competitive, sandwich and steric inhibition immunoassay techniques are useful. The competitive and sandwich methods employ a phase-separation step as an integral part of the method, while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of CT-1 and for substances that bind CT-1, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell-surface receptors, or antigens.

Analytical methods for CT-1 or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner, and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label CT-1 nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminscent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I; fluorophores such as rare earth chelates or fluorescein and its derivatives; rhodamine and its derivatives; dansyl; umbelliferone; luciferases, *e.g.*, firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456); luciferin; 2,3-dihydrophthalazinediones; malate dehydrogenase; urease; peroxidase such as horseradish peroxidase (HRP); alkaline phosphatase; β-galactosidase; glucoamylase; lysozyme; saccharide oxidases, *e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase; heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase; biotin/avidin; spin labels; bacteriophage labels; stable free radicals; and the like.

Those of ordinary skill in the art will know of other suitable labels that may be employed in accordance with the present invention. The binding of these labels to CT-1, antibodies, or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the polypeptide with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Patent Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter *et al.*, Nature, 144: 945 (1962); David *et al.*, Biochemistry, 13: 1014-1021 (1974); Pain *et al.*, J. Immunol. Methods, 40: 219-230 (1981); Nygren, J. Histochem. and Cytochem., 30: 407-412 (1982); O'Sullivan *et al.*, "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166; Kennedy *et al.*, Clin. Chim. Acta, 70: 1-31 (1976); and Schurs *et al.*, Clin. Chim. Acta, 81: 1-40 (1977). Coupling techniques mentioned in the lattermost reference are the glutaraldehyde method, the periodate method, the dimaleimide method, and the m-maleimidobenzyl-N-hydroxysuccinimide ester method.

In the practice of the present invention, enzyme labels are a preferred embodiment. No single enzyme is ideal for use as a label in every conceivable assay. Instead, one must determine which enzyme is suitable for a particular assay system. Criteria important for the choice of enzymes are turnover number of the pure enzyme (the number of substrate molecules converted to product per enzyme site per unit of time), purity of the enzyme preparation, sensitivity of detection of its product, ease and speed of detection of the enzyme reaction, absence of interfering factors or of enzyme-like activity in the test fluid, stability of the enzyme and its conjugate, availability and cost of the enzyme and its conjugate, and the like. Included among the enzymes used as preferred labels in the assays of the present invention are alkaline phosphatase, HRP, beta-galactosidase, urease, glucose oxidase, glucoamylase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase. Urease is

among the more preferred enzyme labels, particularly because of chromogenic pH indicators that make its activity readily visible to the naked eye.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*, U.S. Patent No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, *e.g.*, by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte, the presence of the anti-analyte modifies the enzyme activity. In this case, CT-1 or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with anti-CT-1 so that binding of the anti-CT-1 inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, *e.g.*, a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of CT-1 or CT-1 antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-CT-1 monoclonal antibody as one antibody and a polyclonal anti-CT-1 antibody as the other is useful in testing samples for CT-1 activity.

The foregoing are merely exemplary diagnostic assays for CT-1 and antibodies. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

Identification and *In Vitro* Activity of a CT-1

A. Assay for Expression-Cloned Material

The assay used for hypertrophy is an *in vitro* neonatal rat heart hypertrophy assay described in general as follows:

1. Preparation of the Myocyte Cell Suspension

The preparation of the myocyte cell suspension is based on methods outlined in Chien *et al.*, J. Clin. Invest., 75: 1770-1780 (1985) and Iwaki *et al.*, *supra*. Ventricles from the hearts of 1-2 day Sprague-Dawley rat pups were removed and trisected. The minced ventricles were digested with a series of sequential collagenase treatments. Purification of the resulting single-cell suspension on a discontinuous Percoll gradient resulted in a suspension of 95% pure myocytes.

2. Plating and Culture of the Myocytes

Two published methods for plating and culturing the myocytes are as follows: (1) Long *et al.*, *supra*, preplated the cell suspension for 30 min. in MEM/5% calf serum. The unattached myocytes were then plated in serum-free MEM supplemented with insulin, transferrin, BrdU, and bovine serum albumin in 35-mm tissue-culture dishes at a density of 7.5×10^4 cells per mL. (2) Iwaki *et al.*, *supra*, plated the cell suspension in D-MEM/199/5% horse serum/5% fetal calf serum in 10-cm tissue-culture dishes at 3×10^5 cells per mL. After 24 hr in culture the cells were washed and incubated in serum-free D-MEM/199.

A different protocol has been developed in accordance with this invention for plating and culturing these cells to increase testing capacity with a miniaturized assay. The wells of 96-well tissue-culture plates are precoated with D-MEM/F12/4% fetal calf serum for 8 hr at 37°C. This medium is removed and the cell suspension is plated in the inner 60 wells at 7.5×10^4 cells per mL in D-MEM/F-12 supplemented with insulin, transferrin, and aprotinin. The medium typically also contains an antibiotic such as penicillin/streptomycin and glutamine. This medium allows these cells to survive at this low plating density without serum. Test substances are added directly into the wells after the cells have been in culture for 24 hours.

3. Readout of Hypertrophy

After stimulation with alpha adrenergic agonists or endothelin, neonatal rat myocardial cells in culture display several features of the *in vivo* cardiac muscle cell hypertrophy seen in congestive heart failure, including an increase in cell size and an increase in the assembly of an individual contractile protein into organized contractile units. Chien *et al.*, FASEB J., *supra*. These changes can be viewed with an inverted phase microscope and the degree of hypertrophy scored with an arbitrary scale of 7 to 0, with 7 being fully hypertrophied cells and 3 being non-stimulated cells. The 3 and 7 states may be seen in Simpson *et al.*, Circulation Research, 51: 787-801 (1982), Figure 2, A and B, respectively. To facilitate the microscopic readout of the 96-well cultures and to generate a permanent record, the myocytes are fixed and stained after the

appropriate testing period with crystal violet stain in methanol. Crystal violet is a commonly used protein stain for cultured cells.

Additionally, an aliquot can be taken from the 96-well plates and monitored for the expression of protein markers of the response such as release of ANF or ANP.

B. Expression Cloning

Poly(A)$^+$ RNA was isolated (Aviv and Leder, Proc. Natl. Acad. Sci. USA, 69: 1408-1412 (1972); Cathala *et al.*, DNA, 2: 329-335 (1983)) from day 7 mouse embryoid bodies. Embryoid bodies were generated by the differentiation of pluripotent embryonic stem (ES) cells (Doetschman *et al.*, J. Embryol. Exp. Morphol., 87: 27-45 (1985)). The embryonic stem cell line ES-D3 (ATCC No. CRL 1934) was maintained in an undifferentiated state in a medium containing LIF (Williams *et al.*, Nature, 336: 684-687 (1988)). This medium contained D-MEM (high glucose), 1% glutamine, 0.1 mM 2-mercaptoethanol, penicillin-streptomycin, 15% heat-inactivated fetal bovine serum, and 15 ng/mL mouse LIF. When these cells were put into suspension culture in the same medium without LIF and containing 20% heat-inactivated fetal bovine serum (day 0), they aggregated and differentiated into multicellular structures called embryoid bodies. By day 8 of culture, beating primordial heart-like structures formed on a fraction of the bodies. The embryoid bodies were evaluated for the production of CT-1 activity by changing the differentiating ES cells to serum-free medium (D-MEM/F-12, 1% glutamine, penicillin-streptomycin, containing 0.03% bovine serum albumin) for a 24-hour accumulation. Prior to assay, the conditioned medium was concentrated 10 fold with a 3-K ultrafiltration membrane (Amicon), and dialyzed against assay medium. Medium conditioned for 24 hours starting at day 3 gave a hypertrophy score of 4.5-5.5, and starting at day 6 a score of 5.5-7.5.

A cDNA library in the plasmid expression vector, pRK5B (Holmes *et al.*, Science, 253: 1278-1280 (1991)), was prepared following a vector priming strategy (Strathdee *et al.*, Nature, 356: 763-767 (1992)). The vector, pRK5B, was linearized at the *Not*I site, treated with alkaline phosphatase, and ligated to the single-stranded oligonucleotide, ocdl.1.3, having the sequence:

5'-GCGGCCGCGAGCTCGAATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTT (SEQ ID NO: 5). The ligated product was then cut with *Bst*XI, and the 4700-bp vector fragment was isolated by agarose gel electrophoresis. The vector was further purified by oligo dA chromatography.

The expression library was constructed using 1 μg of the poly (A)$^+$ RNA, 5 μg of vector primer, and reagents from Amersham. Following first- and second-strand synthesis and T4 DNA polymerase fill-in reactions, the material was sized for inserts of greater than 500 bp by gel electrophoresis and circularized by blunt-end ligation without the addition of linkers. The ligations were used to transform *E. coli* strain DH5α by electroporation. From 1 μg of poly(A)$^+$ RNA, 499 ng of double-stranded cDNA were generated. Seventeen nanograms of cDNA were ligated, and 3.3 ng were transformed to yield 780,000 clones, 83% of which had inserts with an average size of 1470 bp.

DNA was isolated from pools of 75-15,000 clones and transfected into human embryonic kidney 293 cells by Lipofectamine transfection (Gibco BRL). Two micrograms of DNA were used to transfect ~200,000 cells in 6-well dishes. The cells were incubated in 2 mL of serum-free assay medium for four days. This medium consisted of 100 mL D-MEM/F-12, 100 μL transferrin (10 mg/mL), 20 μL insulin (5 mg/mL), 50 μL aprotinin (2 mg/mL), 1 mL pen/strep (JRH Biosciences No. 59602-77P), and 1 mL L-glutamine (200 mM). Transfection

and expression efficiency was monitored by the inclusion of 0.2 μg of DNA for a plasmid expressing a secreted form of alkaline phosphatase (Tate *et al.*, FASEB J., 4: 227-231 (1990)).

One hundred microliters of conditioned culture medium from each transfected pool was assayed for hypertrophy in a final volume of 200 μL. For some pools the conditioned medium was concentrated 4-5 fold before assay with Centricon 3™ microconcentrators (Amicon). Ninety pools of 10,000-15,000 clones, 359 pools of 1000-5800 clones, and 723 pools of 75-700 clones were transfected and assayed for hypertrophy activity. Of these 1172 pools, two were found to be positive. Pool 437 (a pool of 187 clones) and pool 781 (a pool of 700 clones) gave scores of 4. A pure clone (designated pchf.437.48) from pool 437 was isolated by retransfection of positive pools containing fewer and fewer numbers of clones until a single clone was obtained. A pure clone from pool 781 (designated pchf.781) was isolated by colony hybridization to the insert from clone pchf.437.48.

The sequence for the insert of clone pchf.781 is provided in Figure 1 (SEQ ID NOS: 1, 2, and 3 for the two nucleotide strands and amino acid sequence, respectively). The sequence of the insert of clone pchf.437.48 matches clone 781 starting at base 27 (underlined).

The first open reading frame of clone pchf.781 (see translation, Fig. 1) encodes a protein of 203 amino acids (translated MW = 21.5 kDa). This protein contains one cysteine residue, one potential N-linked glycosylation site, and no hydrophobic N-terminal secretion signal sequence. The 3' untranslated region of clone pchf.781 contains a common mouse repeat known as b1 (bp ~895-1015). Hybridization of 7-day embryoid body poly(A)$^+$ RNA with a probe from clone pchf.781 shows a single band of ~1.4 kb, which is about the same size as the insert from the cDNA clones.

The encoded sequence is not highly similar (> 35% amino acid identity) to any known protein sequences in the Dayhoff database. It does, however, show a low degree of similarity to a family of distantly related proteins including CNTF, interleukin-6 (IL-6), interleukin-11 (IL-11), LIF, and oncostatin M (OSM) (Bazan, Neuron, 7: 197-208 (1991)). Mouse CT-1 has 24% amino acid identity with mouse LIF (Rose and Todaro, WO 93/05169) and 21% amino acid identity with human CNTF (McDonald *et al.*, Biochim. Biophys. Acta, 1090: 70-80 (1991)). See Figure 2 for an alignment of mouse CT-1 and human CNTF sequences. CNTF, IL-6, IL-11, LIF, and OSM use related receptor signaling proteins including gp130 that are members of the GH/cytokine receptor family (Kishimoto *et al.*, Cell, 76: 253-262 (1994)). CNTF, like CT-1, lacks an N-terminal secretion signal sequence.

C. Identity and Activity of Clone

To demonstrate that clone pchf.781 encodes a CT-1, expression studies were performed both by transfection of 293 cells and by utilizing a coupled *in vitro* SP6 transcription/translation system. ^{35}S-methionine and cysteine labeling of the proteins produced by pchf.781-transfected 293 cells (in comparison with vector-transfected cells) showed that the conditioned medium contained a labeled protein of about 21.8 kDa, and that the cell extract showed a protein of 22.5 kDa. Conditioned media from these transfections gave a morphology score of 6 when assayed for cardiac hypertrophy at a dilution of 1:4 using the assay described above. Conditioned media from unlabeled transfections gave a morphology score of 5.5-6.5 at a dilution of 1:1.

These assays were also positive for a second measure of cardiac hypertrophy--ANP release. See Figure 3. This assay was performed by determination of the competition for the binding of ^{125}I-rat ANP for a rat ANP receptor A-IgG fusion protein. This method is similar to that used for the determination of gp120 using a CD4-

IgG fusion protein (Chamow *et al.*, Biochemistry, 29: 9885-9891 (1990)). Briefly, microtiter wells were coated with 100 µL of rat anti-human IgG antibody (2 µg/mL) overnight at 4° C. After washing with phosphate-buffered saline containing 0.5% bovine serum albumin, the wells were incubated with 100 µL of 3 ng/mL rat ANP receptor A-IgG (produced and purified in a manner analogous to the human ANP receptor A-IgG (Bennett *et al.*, J. Biol. Chem., 266: 23060-23067 (1991)) for one hour at 24°C. The wells were washed and incubated with 50 µL of rat ANP standard or sample for one hour at 24°C. Then 50 µL of ^{125}I-rat ANP (Amersham) was added for an additional one-hour incubation. The wells were washed and counted to determine the extent of binding competition. ANP concentrations in the samples were determined by comparison to a rat ANP standard curve.

^{35}S-methionine labeling of the proteins made by SP6-coupled *in vitro* transcription/translation (materials from Promega) of clone pchf.781 showed a labeled protein of 22.4 kDa. The labeled translation product was active when assayed for cardiac hypertrophy at a dilution of 1:200 (morphology score 5-6). To verify that the 22.4-kDa-labeled band was responsible for the hypertrophy activity, the labeled translation product was applied to a reverse-phase C4 column (Synchropak RO-4-4000) equilibrated in 10% acetonitrile, 0.1% TFA, and eluted with an acetonitrile gradient. Coincident peaks of labeled protein and hypertrophy activity eluted from this column at ~55% acetonitrile.

A cardiac myocyte hypertrophy activity has been reported and partially purified from rat cardiac fibroblasts. Long *et al.*, *supra*. To investigate further the identity of the CT-1 herein, rat cardiac fibroblasts were cultured. Conditioned medium from these primary cultures does have cardiac hypertrophy in the *in vitro* neonatal rat heart hypertrophy assay herein. Blot hybridization of rat fibroblast mRNA isolated from these cultures shows a clear band of 1.4 kb when probed with a coding region fragment of clone pchf.781. (Hybridization was performed in 5 x SSC, 20% formamide at 42° C with a final wash in 0.2 x SSC at 50° C.)

D. Purification of Factor

The culture medium conditioned by cells transfected with clone pchf.781 or a human clone is adjusted to 1.5 M NaCl and applied to a Toyopearl™ Butyl-650M column. The column is washed with 10 mM TRIS-HCl, pH 7.5, 1 M NaCl, and the activity eluted with 10 mM TRIS-HCl, pH 7.5, 10 mM Zwittergent™ 3-10. The peak of activity is adjusted to 150 mM NaCl, pH 8.0, and applied to a MONO-Q Fast Flow column. The column is washed with 10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.1% octyl glucoside and activity is found in the flow-through fraction. The active material is then applied to a reverse phase C4 column in 0.1% TFA, 10% acetonitrile, and eluted with a gradient of 0.1% TFA up to 80%. The activity fractionates at about 15-30 kDa on gel-filtration columns. It is expected that a chaotrope such as guanidine-HCl is required for resolution and recovery.

EXAMPLE II

Testing for *in vivo* Hypertrophy Activity

A. Normal Rats

The purified CT-1 from Example I is tested in normal rats to observe its effect on cardiovascular parameters such as blood pressure, heart rate, systemic vascular resistance, contractility, force of heart beat, concentric or dilated hypertrophy, left ventricular systolic pressure, left ventricular mean pressure, left ventricular end-diastolic pressure, cardiac output, stroke index, histological parameters, ventricular size, wall thickness, *etc.*

B. Pressure-Overload Mouse Model

The purified CT-1 is also tested in the pressure-overload mouse model wherein the pulmonary artery is constricted, resulting in right ventricular failure.

C. RV Murine Dysfunctional Model

A retroviral murine model of ventricular dysfunction can be used to test the purified CT-1, and the dP/dt, ejection fraction, and volumes can be assayed with the hypertrophy assay described above. In this model, the pulmonary artery of the mouse is constricted so as to generate pulmonary hypertrophy and failure.

D. Transgenic Mouse Model

Transgenic mice that harbor a muscle actin promoter-IGF-1 fusion gene display cardiac and skeletal muscle hypertrophy, without evidence of myopathy or heart failure. Further, IGF-1- gene-targeted mice display defects in cardiac myogenesis (as well as skeletal) including markedly decreased expression of ventricular muscle contractile protein genes. The purified CT-1 is tested in these two models.

Additional genetic-based models of dilated cardiomyopathy and cardiac dysfunction, without necrosis, can be developed in transgenic and gene-targeted mice (MLC-*ras* mice; aortic banding of heterozygous IGF-1-deficient mice).

E. Post-Myocardial Infarction Rat Model

The purified CT-1 is also tested in a post-myocardial infarction rat model, which is predictive of human congestive heart failure in producing natriuretic peptide. Specifically, male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., eight weeks of age) are acclimated to the facility for at least one week before surgery. Rats are fed a pelleted rat chow and water *ad libitum* and housed in a light- and temperature-controlled room.

1. Coronary Arterial Ligation

Myocardial infarction is produced by left coronary arterial ligation as described by Greenen *et al.*, J. Appl. Physiol., 93: 92-96 (1987) and Buttrick *et al.*, Am. J. Physiol., 260: 11473-11479 (1991). The rats are anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally), intubated via tracheotomy, and ventilated by a respirator (Harvard Apparatus Model 683). After a left-sided thoracotomy, the left coronary artery is ligated approximately 2 mm from its origin with a 7-0 silk suture. Sham animals undergo the same procedure except that the suture is passed under the coronary artery and then removed. All rats are handled according to the "Position of the American Heart Association on Research Animal Use" adopted 11 November 1984 by the American Heart Association. Four to six weeks after ligation, myocardial infarction could develop into heart failure in rats.

In clinical patients, myocardial infarction or coronary artery disease is the most common cause of heart failure. Congestive heart failure in this model reasonably mimics congestive heart failure in most human patients.

2. Electrocardiograms

One week after surgery, electrocardiograms are obtained under light metofane anesthesia to document the development of infarcts. The ligated rats of this study are subgrouped according to the depth and persistence of pathological Q waves across the precordial leads. Buttrick *et al.*, *supra*; Kloner *et al.*, Am. Heart J., 51: 1009-1013 (1983). This provides a gross estimate of infarct size and assures that large and small infarcts are not

differently distributed in the ligated rats treated with CT-1 or CT-1 antagonist and vehicle. Confirmation is made by precise infarct size measurement.

3. CT-1 or CT-1 Antagonist Administration

Four weeks after surgery, CT-1 or CT-1 antagonist (10 µg/kg to 10 mg/kg twice a day for 15 days) or saline vehicle is injected subcutaneously in both ligated rats and sham controls. Body weight is measured twice a week during the treatment. CT-1 or CT-1 antagonist is administered in saline or water as a vehicle.

4. Catheterization

After 13-day treatment with CT-1, CT-1 antagonist, or vehicle, rats are anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). A catheter (PE 10 fused with PE 50) filled with heparin-saline solution (50/U/mL) is implanted into the abdominal aorta through the right femoral artery for measurement of arterial pressure and heart rate. A second catheter (PE 50) is implanted into the right atrium through the right jugular vein for measurement of right atrial pressure and for saline injection. For measurement of left ventricular pressures and contractility (dP/dt), a third catheter (PE 50) is implanted into the left ventricle through the right carotid artery. For the measurement of cardiac output by a thermodilution method, a thermistor catheter (Lyons Medical Instrument Co., Sylmar, CA) is inserted into the aortic arch. The catheters are exteriorized at the back of the neck with the aid of a stainless-steel wire tunneled subcutaneously and then fixed. Following catheter implantation, all rats are housed individually.

5. Hemodynamic Measurements

One day after catherization, the thermistor catheter is processed in a microcomputer system (Lyons Medical Instrument Co.) for cardiac output determination, and the other three catheters are connected to a Model CP-10 pressure transducer (Century Technology Company, Inglewood, CA) coupled to a Grass Model 7 polygraph (Grass Instruments, Quincy, MA). Mean arterial pressure (MAP), systolic arterial pressure (SAP), heart rate (HR), right atrial pressure (RAP), left ventricular systolic pressure (LVSP), left ventricular mean pressure (LVMP), left ventricular end-diastolic pressure (LVEDP), and left ventricular maximum (dP/dt) are measured in conscious, unrestrained rats.

For measurement of cardiac output, 0.1 mL of isotonic saline at room temperature is injected as a bolus via the jugular vein catheter. The thermodilution curve is monitored by VR-16 simultrace recorders (Honeywell Co., NY) and cardiac output (CO) is digitally obtained by the microcomputer. Stroke volume (SV)=CO/HR; Cardiac index (CI)=CO/BW; Systemic vascular resistance (SVR)=MAP/CI.

After measurement of these hemodynamic parameters, 1 mL of blood is collected through the arterial catheter. Serum is separated and stored at -70° C for measurement of CT-1 levels or various biochemical parameters if desired.

At the conclusion of the experiments, the rats are anesthetized with pentobarbital sodium (60 mg/kg) and the heart is arrested in diastole with intra-atrial injection of KCl (1 M). The heart is removed, and the atria and great vessels are trimmed from the ventricle. The ventricle is weighed and fixed in 10% buffered formalin.

All experimental procedures are approved by the Institutional Animal Care and Use Committee of Genentech, Inc. before initiation of the study.

6. Infarct Size Measurements

The right ventricular free wall is dissected from the left ventricle. The left ventricle is cut in four transverse slices from apex to base. Five micrometer sections are cut and stained with Massons' trichrome stain and mounted. The endocardial and epicardial circumferences of the infarcted and non-infarcted left ventricle are determined with a planimeter Digital Image Analyzer. The infarcted circumference and the left ventricular circumference of all four slices are summed separately for each of the epicardial and endocardial surfaces and the sums are expressed as a ratio of infarcted circumference to left ventricular circumference for each surface. These two ratios are then averaged and expressed as a percentage for infarct size.

7. Statistical Analysis

Results are expressed as mean ± SEM. Two-way and one-way analysis of variance (ANOVA) is performed to assess differences in parameters among groups. Significant differences are then subjected to post hoc analysis using the Newman-Keuls method.

$p < 0.05$ is considered significant.

8. Results

The mean body weight before and after treatment with CT-1 or CT-1 antagonist or vehicle is not expected to be different among the experimental groups. Infarct size in ligated rats is not expected to differ between the vehicle-treated group and the CT-1- or CT-1- antagonist-treated group.

It is expected that administration of CT-1 or CT-1 antagonist to the ligated rats in the doses set forth above would result in improved cardiac hypertrophy by increasing ventricular contractility and decreasing peripheral vascular resistance over that observed with the vehicle-treated sham and ligated rat controls. This expected result would demonstrate that administration of CT-1 or CT-1 antagonist improves cardiac function in congestive heart failure. In sham rats, however, CT-1 or CT-1 antagonist administration at this dose is not expected to alter significantly cardiac function except possibly slightly lowering arterial pressure and peripheral vascular resistance.

It would be reasonably expected that the rat data herein may be extrapolated to horses, cows, humans, and other mammals, correcting for the body weight of the mammal in accordance with recognized veterinary and clinical procedures. Using standard protocols and procedures, the veterinarian or clinician will be able to adjust the doses, scheduling, and mode of administration of CT-1 or a CT-1 antagonist to achieve maximal effects in the desired mammal being treated. Humans are believed to respond in this manner as well.

EXAMPLE III

Proposed Clinical Treatment of Dilated Cardiomyopathy

A. Intervention

Patient self-administration of CT-1 or CT-1 antagonist at an initial dose of 10-150 μg/kg/day is proposed. The dose would be adjusted downward for adverse effects. If no beneficial effects and no limiting adverse effects are determined at the time of re-evaluation, the dose would be adjusted upward. Concurrent medication doses (*e.g.*, captopril as an ACE inhibitor and diuretics) would be adjusted at the discretion of the study physician. After the maximum dose is administered for 8 weeks, the CT-1 or CT-1 antagonist administration is stopped, and re-evaluation is performed after a similar time period off treatment (or a placebo).

B. Inclusion Criteria

Patients would be considered for the study if they meet the following criteria:

-Dilated cardiomyopathy (DCM). Idiopathic DCM, or ischemic DCM without discrete areas of akinesis dyskinesis of the left ventricle (LV) on contrast ventriculography or 2D echocardiography. Evidence for impaired systolic function to include either LV end-diastolic dimension (EDD) > 3.2 cm/m^2 BSA or EDV > 82 mL/m^2 on 2D echocardiography, LV fractional shortening < 28% on echocardiography, or ejection fraction (by contrast ventriculography or radionuclide angiography) < 0.49.

-Symptoms. New York Heart Association class III or peak exercise VO_2 < 16 mL/kg/min. (adjusted for age), stable for at least one month on digoxin, diuretics, and vasodilators (ACE inhibitors).

-Concurrent ACE inhibitor therapy.

-Adequate echocardiographic "windows" to permit assessment of left ventricular volume and mass.

-Ability to self-administer CT-1 or CT-1 antagonist according to the dosage schedule, and to return reliably for follow-up assessments.

-Consent of patient and patient's primary physician to participate.

-Absence of exclusion criteria.

C. Exclusion Criteria

Patients would be excluded from consideration for any of the following reasons:

-Dilated cardiomyopathy resulting from valvular heart disease (operable or not), specific treatable etiologies (including alcohol, if abstinence has not been attempted), or operable coronary artery disease.

-Exercise limited by chest pain or obstructive peripheral vascular disease.

-Chronic obstructive lung disease.

-Diabetes mellitus or impaired glucose tolerance.

-History of carpal tunnel syndrome, or evidence for positive Tinel's sign on examination.

-History of kidney stones.

-Symptomatic osteoarthritis.

-Inability to consent for or participate in serial bicycle ergometry with invasive hemodynamic monitoring (as described below).

-Active malignancy.

D. Patient Assessment

1) Major Assessment Points: baseline; after peak stable CT-1 or CT-1 antagonist dose maintained for 8 weeks; after equal period after drug discontinuation.

-It is anticipated that patients would remain in the hospital for two to three days at the onset of active treatment, with daily weights and laboratory data including electrolytes, phosphorus, BUN, creatinine, and glucose. Following this, they would be monitored on the Clinical Research Center floor daily for an additional two to three days.

i. Physical examination.

ii. Symptom Point Score (Kelly *et al.*, Amer. Heart J., 119: 1111 (1990)).

iii. Laboratory data: CBC; electrolytes (including Mg^{+2} and Ca^{+2}); BUN; creatinine; phosphorus; fasting glucose and lipid profile (total cholesterol, HDL-C, LDL-C, triglycerides); liver function tests (AST, ALT, alkaline phosphatase, total bilirubin); total protein; albumin; uric acid; and CT-1.

iv. 2D, M-mode, and doppler echocardiography, including: diastolic and systolic dimensions at the papillary muscle level; ejection fraction estimate by area planimetry from apical 2-chamber and 4-chamber views, estimated systolic and diastolic volumes by Simpson's rule method, and estimated left ventricular mass; doppler assessment of mitral valve inflow profile (IVRT, peak E, peak A, deceleration time, A wave duration), and pulmonary vein flow profile (systolic flow area, diastolic flow area, A reversal duration, and velocity).

v. Rest and exercise hemodynamics and measured oxygen consumption, using bicycle ergometry with percutaneously inserted pulmonary artery and arterial catheters. Perceived exertion level would be scored on the Borg scale, and measurements of pulmonary artery systolic, diastolic, and mean pressures, as well as arterial pressures and pulmonary capillary wedge pressure would be measured at each increment of workload, along with arterial and mixed venous oxygen content for calculating cardiac output.

vi. Assessment of body fat and lean body mass, as well as skeletal muscle strength and endurance.

2) Interim Assessment Points: weekly

i. Physical examination.

ii. Symptom Point Score.

iii. Laboratory data: electrolytes, BUN, creatinine, phosphorus, fasting glucose, somatomedin-C, and CT-1.

E. Potential Benefits

1) Improved sense of well-being.

2) Increased exercise tolerance.

3) Increased muscle strength and lean body mass.

4) Decreased systemic vascular resistance.

5) Enhanced cardiac performance.

6) Enhanced compensatory myocardial hypertrophy.

EXAMPLE IV

Testing for *in vitro* Neurotrophic Activity

An assay used for ciliary ganglion neurotrophic activity was performed as described in Leung, Neuron, 8: 1045-1053 (1992). Briefly, ciliary ganglia were dissected from E7-E8 chick embryos and dissociated in trypsin-EDTA (Gibco 15400-013) diluted ten fold in phosphate-buffered saline for 15 minutes at 37° C. The ganglia were washed free of trypsin with three washes of growth medium (high glucose D-MEM supplemented with 10% fetal bovine serum, 1.5 mM glutamine, 100 µg/mL penicillin, and 100 µg/mL strepomycin), and then gently triturated in 1 mL of growth medium into a single-cell suspension. Neurons were enriched by plating this cell mixture in 5 mL of growth media onto a 100-mm tissue culture dish for 4 hours at 37° C in a tissue culture incubator. During this time the non-neuronal cells preferentially stuck to the dish and neurons were gently washed free at the end of the incubation.

The enriched neurons were then plated into a 96-well plate previously coated with collagen. In each well, 1000 to 2000 cells were plated, in a final volume of 100 to 250 µL, with dilutions of the conditioned medium from the pchf.781-transfected 293 cells of Example I. The cells were also plated with the transfected 293 conditioned medium as a control, and with a CNTF standard as a comparison. Following a 2-4-day incubation at 37° C, the number of live cells was assessed by staining live cells using the vital dye metallothionine (MTT). One-fifth of the volume of 5 mg/mL MTT (Sigma M2128) was added to the wells. After a 2-4-hour incubation at 37° C, live cells (filled with a dense purple precipitate) were counted by phase microscopy at 100X magnification.

The results of the assay are shown in Figure 4. It can be seen that the pchf.781 transfection (triangles) increased survival of the live neurons (measured by cell count) as the fraction of assay volume of transfected 293 conditioned medium increased. This is similar to the pattern for the CNTF standard (circles), and is in contrast to the control transfection (squares), which showed no increase in survival as a function of increased fraction of assay volume of conditioned medium. This indicates that CT-1 is useful as a neurotrophic agent, having a similar effect to that observed with CNTF.

EXAMPLE V

A source of mRNA encoding human CT-1 (also known as human cardiotrophin-1 (CT-1)) was identified by screening poly(A)+RNA from several adult tissues with a probe from the mouse CT-1 cDNA clones. Heart, skeletal muscle, colon, ovary, and prostate showed a 1.8 kb band upon blot hybridization with a 180-bp mouse CT-1 probe (extending from 19 bp 5' of the initiating ATG through amino acid 50) in 20% formamide, 5 X SSC at 42° C with a final wash at 0.25 X SSC at 52° C. Clones encoding human CT-1 were isolated by screening a human heart cDNA library (Clontech) with the same probe and conditions (final wash at 55° C).

Eleven clones were isolated from 1 million screened. The *Eco*RI inserts of several of the clones were subcloned into plasmid vectors and their DNA sequences determined.

The DNA sequence from clone h5 (SEQ ID NOS: 6 and 7 for the sense and anti-sense strands, respectively) is shown in Figure 5 and includes the whole coding region. Clone h5 (pBSSK+.hu.CT1.h5) was deposited on July 26, 1994 in the American Type Culture Collection as ATCC No. 75,841. The DNA sequence of another clone, designated h6, matches that of clone h5 in the region of overlap. Clone h6 begins at base 47 of clone h5 and extends 3' of clone h5 for an additinal 521 bases. The encoded protein sequence of human CT-1 (SEQ ID NO: 8) is 79% identical with the mouse CT-1 sequence (SEQ ID NO: 3), as evident from Figure 6, wherein the former is designated "humct1" and the latter is designated "chf.781."

To show that human CT-1 encoded by clone h5 is biologically active, the *Eco*RI fragment was cloned into the mammalian expression vector pRK5 (EP 307,247) at the unique *Eco*RI site to give the plasmid pRK5.hu.CT1. This plasmid was transfected into human 293 cells, and the cells were maintained in serum-free medium for 3-4 days. This medium was then assayed for cardiac myocyte hypertrophy as described above for mouse CT-1. The transfected 293 conditioned medium was clearly active in this assay (hypertrophy score of 5.5 at a dilution of 1:20; Table 3). Other cytokines were also tested for hypertrophy activity (Table 3).

Table 3

Hypertrophy assay of CT-1-related cytokines

Cytokine	Conc., nM	Hypertrophy Score*
None	0	3
CT-1 fusion	0.05	6
	0.1	5
	0.25	6
	0.5	6.5
	1.0	7
Mouse LIF	0.05	4
	0.25	5.5
	2.5	6
Human IL-11	0.1	3.5
	0.2	4.5
	0.5	4.5
	1.0	4.5
	2.0	5.5
Human OSM	6.25	4.5
	12.5	4.5
	25	5
	50	6
Mouse IL-6	50	3.5
	100	3.5
Rat CNTF	25	4
	100	4

*A score of 3 is no hypertrophy; 7 is maximal hypertrophy (see *Materials and Methods*).

The mouse and human CT-1 encoded by these clones have 80% amino acid identity and are about 200 amino acids in length corresponding to a calculated molecular mass of 21.5 kDA. Both human and mouse CT-1 lack a conventional hydrophobic amino terminal secretion sequence, however, they are found in the medium of transfected mammalian cells. The coding regions of human and mouse CT-1 are contained on three separate exons that span 6-7 kbp of genomic DNA. The human CT-1 gene was localized by fluorescent *in situ* hybridization and by somatic cell hybridization to chromosome 16p11.1 - p11.2.

The expression pattern of mouse CT-1 was determined by Northern blot analysis. CT-1 mRNA is widely (but not universally) expressed in adult mouse tissues including heart, kidney, skeletal muscle, and liver. A single 1.4 kb CT-1 mRNA species was detected in the adult mouse heart, skeletal muscle, liver, lung, and kidney. Lower amounts of mRNA were seen in testis and brain, while no expression was observed in the spleen. The CT-1 transcript was also detected in seven-day embryoid body mRNA, which was the RNA used

to prepare the cDNA expression library. In a survey of human adult tissues (Figure 20), high levels of CT-1 mRNA (1.7 kb mRNA) were seen in heart, skeletal muscle, prostate and ovary. Lower levels were observed in lung, kidney, pancreas, thymus, testis and small intestine. Little or no expression was seen in the brain, placenta, liver spleen, colon or peripheral blood leukocytes. High levels of expression were also seen in human fetal heart, lung, and kidney, suggesting that CT-1 might be involved in embryonic development of these organs. *In situ* analysis of CT-1 expression during mouse embryogenesis reveals widespread expression in a variety of non-cardiac systems. The high level of expression in these other adult tissues suggests the possibility of functional roles for CT-1 in a wide variety of adult organ systems, outside of the cardiovascular system. The pattern in humans and mouse are similar with the exception of expression in the liver, which is weakly positive in human samples.

Like CNTF, CT-1 lacks a conventional amino-terminal secretion signal sequence; it is, however, found in the medium of transfected mammalian cells.

The predicted tertiary structure of CT-1 is consistent with its containing four amphipathic helices that are features of a large number of cytokines and other proteins including growth hormone. (For reference see Abdel-Meguid *et al., Proc. Natl Acad. Sci. USA*, 84:6434-6437 (1987) and Bazan, *Neuron*, 7:197-208 (1991)). Although these cytokines share biological activities and receptor subunits, alignment of the amino acid sequence of human CT-1 and other members of the IL-6 cytokine family, reveals that they are only distantly related in primary sequence (15%-25% identity) Figure 16. There is little conservation of the cysteine residues and only a partial maintenance of the exon-intron boundaries. Based on the sequence identity comparison determined herein, studies analyzing the crystal structure and biological function of mouse LIF and their relevance to receptor binding (Robinson *et al., Cell*, 77:1101-1116 (1994)) suggest useful subunit regions of CT-1. As determined by X-ray crystallography at a 2.0 Å resolution, the main chain fold of mouse LIF conforms to the four α-helix bundle topography that has been noted for other members of the IL-6 cytokine family. Alignment of the sequences for functionally-related molecules, such as oncostatin M and CNTF, and consequent mapping to the LIF structure, indicated regions of conserved surface character. A series of human and mouse LIF chimeras have identified the fourth helix and the preceding loop as potentially important sites for interaction with the LIF receptor (Robinson *et al., Cell*, 77:1101-1116 (1994)). Although LIF and CT-1 display a high degree of divergence in primary sequence within these regions, the similar domains within CT-1 are likely important in maintaining the interactions of CT-1 with the LIF receptor. Peptides derived from these regions will find use as CT-1 agonists (see Figure 16 for example). Similar approaches to generate mouse LIF/CT-1 chimeras will be of value.

Human CT-1 binds to the mouse LIF receptor. As discussed herein, human CT-1 was expressed by subcloning the coding region from plasmid pBSSK+.hu.CT1.h5, which contained all of the cDNA protein coding

region, to give plasmid pRK5.hu.CT1. Clarified conditioned medium was obtained from human 293 cells transfected with this plasmid and maintained in serum-free medium for four days. Binding to M1 cells (ATCC TIB 192), Hela cells and WI-26 VA4 (ATCC CCL-95.1) cells was performed for 2 hours a 4 degrees C and analyzed as described herein. For the Hela cell binding, CM was concentrated 10 fold and added at a 3-fold dilution to the binding assays. For the WI-26 binding the conditioned medium was used without concentration. This conditioned medium competed for labeled human LIF (iodinated with IODO-BEAD from Pierce or lactoperoxidase methods to a specific activity of 1000-1500 Ci/mmol as described herein) as did purified mouse and human LIF and mouse CT-1. CM from vector transformed cells failed to compete (Figure 17A). While booth mouse and human LIF bind and activate the mouse LIF receptor, mouse LIF fails to bind the human LIF receptor. As shown herein, human LIF competes for the binding of labeled human LIF to Hela cells while mouse LIF does not (Figure 17B). Mouse CT-1 and conditioned medium form 293 cells transfected with the human CT-1 expression vector compete for this binding as well. (Figure 17B). However, the binding of labeled mouse CT-1 is completely competed by unlabeled human LIF. Thus, both human and mouse CT-1 bind to human LIF receptor, and CT-1 lacks the species specificity of binding found for LIF. The affinity of mouse CT-1 for human LIF receptor was determined (Figure 18). A single binding component was observed with an affinity (Kd approx. 0.75 nM), about equal to that for the mouse LIF receptor as shown herein.

Human CT-1 does not bind the specific OSM Receptor. Although oncostatin M binds and functions via the LIF receptor (Gearing et al. (1992) *New Biologist* 4:61-65), but as shown herein CT-1 is not a ligand for the OSM specific receptor, the oncostatin M receptor, which has been identified in and cloned from the human lung cell line WI-26 VA4. Both purified mouse CT-1 and the CM from 293 cells transfected with human CT-1 cDNA failed to compete for labeled OSM binding (Figure 19).

CT-1 induces a distinct form of myocardial cell hypertrophy characterized by sarcomeric assembly in series. The CT-1 induced hypertrophic phenotype is distinct from the hypertrophic phenotype observed following G-protein dependent stimulation with α-adrenergic agonists (Knowlton *et al. (Journal of Biological Chemistry*, 266:7759-7768 (1991); Knowlton *et al., Journal of Biological Chemistry*, 268:15374-15380 (1993), endothelin-1, Shubeita *et al., Journal of Biological Chemistry*, 265:20555-20562 (1990), and angiotensin II (Sadoshima *et al., Circ. Res.*, 73:413-423 (1993)). On a single cell level, heterotrimeric G-protein dependent pathways induce a form of hypertrophy with a relatively uniform increase in myocyte size and the addition of new myofibrils in parallel (Knowlton *et al., Journal of Biological Chemistry*, 268:15374-15380 (1993); Shubeita *et al., Journal of Biological Chemistry*, 265:20555-20562 (1990); Iwaki *et al., Journal of Biological Chemistry*, 265, 13809-13817 (1990)). In contrast, CT-1 induces an increase in myocyte size characterized by a marked increase in cell length, but little or no change in cell width. Consistent with the results presented herein for CT-1. LIF is also capable of activating a similar pattern of hypertrophy in the cultured myocardial cell assay

system, while IL-6 and CNTF had little effect, presumably because of the lack of expression of the private receptor in cultured myocardial cells. LIF signals through the gp130/LIFRβ complex, through which CT-1 also functions as shown herein.

To characterize the effects of gp130/LIFRβ-dependent stimulation on the myofibrillar cytoarchitecture, cardiomyocytes were dual-stained for thick (βMHC) and thin (F-actin) myofilaments, and viewed by confocal laser microscopy (Messerli *et al., Histochemistry,* 100:193-202 (1993)). Cardiomyocytes stimulated with CT-1 and LIF displayed a high degree of myofibrillar organization: myofibrils were organized in a strictly sarcomeric pattern, oriented along the longitudinal cell axis, and extended to the cell periphery. Importantly, the increase in cell size and length was not accompanied by a change in the average sarcomere length, strongly suggesting that the cell elongation in response to gp130/LIFRβ-stimulation results from an addition of new sarcomeric units in series. The morphologic changes induced by gp130/LIFRβ dependent stimulation *in vitro* are reminiscent of the changes observed in cardiac myocytes isolated from hearts subjected to chronic volume overload (Anversa *et al., Circ Res.*, 52:57-64 (1983); Gerdes *et al., Lab Invest.*, 59:857-861 (1988)). By contrast, the pattern of cardiomyocyte hypertrophy induced by α-adrenergic stimulation more closely resembles a pressure overload-like phenotype (Morkin, *Science*, 167:1499-1501 (1970); Anversa *et al., J. Am. Coll. Cardiol.*, 7:1140-1149 (1986)).

On a molecular level, gp130 dependent stimulation and α-adrenergic stimulation result in distinct patterns of embryonic gene, MLC-2v, and immediate early gene expression. The reactivation of an embryonic pattern of gene expression is a central feature of cardiomyocyte hypertrophy (Chien *et al., Faseb J*, 5:5037-3046 (1991)). Members of the embryonic gene program, such as ANF and skeletal α-actin are abundantly expressed in the ventricular myocardium during embryonic development, but their expression is down-regulated shortly after birth. Stimulation of cardiomyocytes with CT-1 or LIF induced prepro-ANF mRNA expression, and perinuclear accumulation and secretion of immunoreactive ANF. However, in contrast to α-adrenergic stimulation, CT-1 and LIF did not induce skeletal α-actin expression. Growth factors, signaling through G-protein coupled receptors, including α-adrenergic agonists, endothelin-1, and angiotensin II, induce ANF and skeletal α-actin in a coordinate fashion (Knowlton *et al., Journal of Biological Chemistry*, 266:7759-7768 (1991); Bishopric *et al., Journal of Clinical Investigation*, 80:1194-1199 (1987); Sadoshima *et al., Circ. Res.*, 73:413-423 (1993)). A recent study compared the expression pattern of distinct members of the embryonic gene program in pressure overload versus volume overload hypertrophy *in vivo* in the rat myocardium (Calderone *et al., Circulation*, 92:2385-2390 (1995)). As shown previously (Izumo *et al., Proc. Natl Acad. Sci. USA*, 85:339-343 (1988)) pressure overload resulted in the coordinate induction of ANF and skeletal α-actin. However, volume overload hypertrophy was associated with a selective increase in ANF expression, and no induction of skeletal α-actin, suggesting that the regulation of distinct embryonic genes *in vivo* is related to the hypertrophic stimulus (Calderone *et al., Circulation*, 92:2385-2390 (1995)). The pattern of embryonic gene expression

induced by CT-1 and LIF in cardiomyocyte culture therefore resembles the pattern observed in volume overload hypertrophy *in vivo*.

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Deposit of Material

The following plasmid has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Plasmid	ATCC Dep. No.	Deposit Date
pBSSK+.hu.CT1.h5	75,841	July 26, 1994

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the plasmid on deposit should die or be lost or destroyed when cultivated under suitable conditions, the plasmid will be promptly replaced on notification with another of the same plasmid. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

EXAMPLE VI

MATERIALS AND METHODS.

Human IL-6 was from Genzyme, mouse LIF was from R & D Systems and Genentech manufacturing, and rat CNTF and GDNF, Poulsen *et al., Neuron*, 13:1245-1252 (1994) were produced by Genentech. Mouse CT-1 was expressed and purified as a fusion protein as described. This protein results in a 34 amino acid N-terminal extension that encodes a portion of the herpes simplex virus glycoprotein D and a factor Xa cleavage

site. In some cases an alternative fusion protein was used that substitutes a different site for the Factor Xa cleavage site giving the amino acid sequence . . . DQLLEGGAAHY followed by the CT-1 sequence MSQREGSL . . . CT-1 and LIF were iodinated by the iodo-bead (Pierce) and lactoperoxidase (Gladek *et al.*, *Arch. Immunol. Ther. Exp.*, 31:541-553 (1983)) methods to specific activities of 900-1100 Ci/mmol.

Hematopoietic, neuronal, and developmental assays. Proliferation of the mouse hybridoma cell line, B9 (Aarden *et al.*, *Eur. J. Immunol.*, 17:1411-1416 (1987)) was assayed by 3H-thymidine incorporation 84 h after the addition of cytokine as described (Nordan *et al.*, *Science*, 233:566-569 (1986)). Inhibition of the proliferation of the mouse myeloblast cell line, M1 (T-22), was assayed by 3H-thymidine incorporation 72 h after the addition of cytokine as described (Lowe *et al.*, *DNA*, 8:351-359 (1989)). The data were fit to the four parameter equation, $y=d-((d-a)/(1+(x/c)^b))$, where the parameter c is the EC_{50}.

For the assay of the transmitter phenotype, newborn rat sympathetic neurons were prepared as described (Hawrot *et al.*, *Meth. Enzymol.*, 58:574-583 (1979)). Superior cervical ganglia were dissociated with trypsin (0.08%) and plated in serum free F-12 medium containing nerve growth factor and additives as described (Davies *et al.*, *Neuron*, 11:565-574 (1993)). Neurons were plated at 30,000 per well in 24 well plates precoated with poly-ornithine and ECL cell attachment matrix (Promega) and allowed to grow for ten days in the presence of indicated factors. Tyrosine hydroxylase and choline acetyltransferase activities were assayed as described (Reinhard *et al.*, *Life Sci.*, 39:2185-2189 (1986); Fonnum, *Biochem. J.*, 115:465-472 (1969)).

The survival of rat dopaminergic neurons was assayed as described (Poulsen *et al.*, *Neuron*, 13:1245-1252 (1994)). Ciliary neuron survival assays were performed with neurons isolated from E8 chick embryos as described (Manthorpe *et al.*, (Rush, R., eds.) Vol. pp. 31-56, John Wiley & Sons (1989)). Survival was assessed by counting live neurons after staining with the vital dye MTT (Mosmann, *J. Immunol. Meth.*, 65:55-63 (1983)). The data were fit to the four parameter equation described above.

For the assay of embryonic stem cell differentiation, passage 15 embryonic stem cells, ES.D3 (Gossler *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:9065-9069 (1986)) were maintained in DMEM (GIBCO, high glucose, no sodium pyruvate), containing 23.83 g/l HEPES, 500 mg/l penicillin, 500 mg/l streptomycin, 4 g/l L-glutamine, 1 g/l gentamicin sulfate, 1 mM 2-mercaptoethanol, 15% fetal bovine serum, and 1.2 Munits/l mouse LIF (GIBCO). Cells were trypsinized, plated in duplicate at 1000 cells per well in 24-well tissue culture plates in the above culture medium with or without LIF or CT-1, and scored 9 days later. No change in colony numbers was observed except in the no addition group where the cells had flattened and differentiated.

Cell binding and cross-linking. Binding was performed in RPMI-1640 containing 0.1 % bovine serum albumin with 7.5-10 million M1 cells (TIB 192, ATCC) in a volume of 250 µl for 2 h on ice with shaking. Reactions were layered on 250 µl of RPMI containing 0.1 % albumin and 20 % sucrose, centrifuged at 4000 rpm

for 1 min at 4° C, and the cell pellet counted. The data were fit to a one-site binding model as described (Munson *et al., Anal. Biochem.*, 107:220-239 (1980)). Lines shown in the figures are from the curve fits.

Anti-gp130 antibody inhibition experiments were performed with a rat anti-mouse gp130 monoclonal antibody (RX435)[3] or a rat anti-gp120 control antibody (Genentech 6D8.1E9) in a volume of 150 µl. Reactions were incubated on ice 2 h, centrifuged at 12,500 rpm, and washed with 1 ml of cold phosphate buffered saline containing 0.1 % albumin. The data were fit to the four parameter equation described above.

Binding to neonatal rat cardiac myocytes was performed as for M1 cells, but cells isolated as described herein and plated for 16 h. Assays were performed with 1 million cells in a volume of 100 µl.

Cross-linking was performed with 10 million M1 cells in phosphate buffered saline containing 0.1% albumin, 7.2 nM ^{125}I-mouse CT-1 or 2.2 nM ^{125}I-mouse LIF, with or without a 100 fold molar excess of the unlabeled ligands in a volume of 250 µl. After 1 h at room temperature, 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 5 mM *N*-hydroxysulfosuccinimide (sulfo-NHS) (Pierce) were added and the incubation continued for 30 min at room temperature. The samples were then processed as described (Greenlund *et al., J. Biol. Chem.*, 268:18103-18110 (1983)).

DNA binding activity. Two hundred thousand M1 cells were incubated in 1 ml of RPMI-1640 in 12-well dishes with ligand for 30 min at 37 C. After stimulation, the cells were collected by centrifugation, suspended in 200 ul of homogenization butter (10 mM HEPES (pH 7.2), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 1 mM phenylmethylsulfonylfluoride, 10 ug/ml leupeptin, 10 ug/ml aprotinin), and incubated at 0 C for 15 min. Cells were lysed by the addition of NP-40 to 0.1%, and cell extracts prepared by incubation at 0 C for 15 min, centrifugation at 100 x g for 5 min, and retention of the supernatant. DNA binding activity in the cell extracts was assayed by electrophoretic mobility shift assay as described (Greenlund *et al., EMBO J.*, 13:1591-1600 (1994)). Briefly, binding reactions contained 10 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 6.7 % glycerol, 0.067 g/l poly(dIdC)(dIdC), 0.5 ng (25,000 cpm) ^{32}P-SIE DNA (5'-CTAGAGTCGACATTTCCCGTAAATCT and 5'-CTAGAGATTTACGGGAAATGTCGACT, high affinity m67 (Sadowski *et al., Science*, 261:1739-1744 (1993); Wagner *et al., EMBO J.*, 9:4477-4484 (1990)), and 3 ul of cell extract in a final volume of 15 ul. Some reactions included 100 ng of unlabeled SIE DNA. The reactions were incubated 30 min at 22 C and analyzed by polyacrylamide gel electrophoresis and autoradiography.

Binding to soluble LIF receptor and soluble gp130. DNA encoding the extracellular domain of the mouse LIF receptor (amino acids 1-826) and mouse gp130 (1-617) was generated by PCR of M1 cell (above) mRNA and of a mouse lung cDNA library (Clontech). These sequences were cloned with a C-terminal tag encoding 6 histidine residues in the mammalian expression vector, pRK5 (Suva *et al., Science*, 237:893-896 (1987)) to give the plasmids, pRK5.mu.slifr and pRK5.mu.sgp130. DNA sequencing of the coding regions confirmed that these plasmids encode proteins that match the published amino acid sequence (Tomida *et al., J.*

Biochem., 115:557-562 (1994); Saito *et al.*, *J. Immunol.*, 148:4066-4071 (1992)), with the exception of the substitution of lysine for arginine at amino acid 326 of gp130, a change that was found for three fragments from both sources. The plasmids were transfected into human 293 cells, and the proteins isolated from 4-day conditioned medium by Ni^{++}-NTA-agarose (Qiagen) affinity purification. Briefly, the conditioned medium was concentrated ~ 18 fold (Centriprep 10, Amicon), and the tagged protein purified by binding to the Ni^{++} resin for 2 h at room temperature. Following two washes with phosphate buffer saline containing 5 mM imidazole, the proteins were eluted with phosphate buffer saline containing 200 mM imidazole and quantitated by colorimetric assay (BioRad). Analysis of the proteins by SDS-polyacrylamide gel electrophoresis showed single bands of 120 kDa for the soluble LIF receptor and 85 kDa for soluble gp130. Amino acid sequencing gave the expected amino terminal sequence for the soluble LIF receptor beginning at amino acid 44 (Tomida *et al.*, *J. Biochem.*, 115:557-562 (1994); von Heijne, *Nucl. Acids Res.*, 14:4683-4690 (1986)); the amino terminus of gp130 is expected to be blocked (Saito *et al.*, *J. Immunol.*, 148:4066-4071 (1992); von Heijne, *Nucl. Acids Res.*, 14:4683-4690 (1986)) and amino terminal protein sequencing gave no sequence for soluble gp130.

Binding to the soluble LIF receptor and soluble gp130 was performed in a manner similar to that previously described (Layton *et al.*, *J. Biol. Chem.*, 269:17048-17055 (1994)). Briefly, assays were performed in 96-well Multiscreen-HV filtration plates with 0.45 µm PVDF membranes (Millipore) in phosphate buffered saline containing 0.1 % bovine serum albumin and including 25 µl of phosphate buffer saline-washed Ni^{++}-NTA-Agarose (Qiagen) in a final volume of 175 µl. Plates were incubated at room temperature overnight with agitation. Following vacuum filtration and one wash with 200 µl of cold phosphate buffer saline, the individual assay wells were cut from the plate and counted. The data were analyzed as described above for M1 binding.

RESULTS

As shown herein some members of the IL-6 cytokine family (LIF, OSM, and IL-11) induce cardiac myocyte hypertrophy *in vitro* like CT-1. The previously known members of this family have a wide range of hematopoietic, neuronal, and developmental activities (Kishimoto *et al.*, *Science*, 258:593-597 (1992)). CT-1 was assayed for its activity in these biological systems.

Hematopoietic assays. IL-6 promotes the proliferation and differentiation of B cells into antibody producing cells following antigen stimulation (Akira *et al.*, *Adv. Immunol.*, 54:1-78 (1993)). In the order to determine whether CT-1 could also mediate these effects, CT-1 was tested on the mouse hybridoma cell line, B9 (Aarden *et al.*, *Eur. J. Immunol.*, 17:1411-1416 (1987)). While IL-6 stimulates the proliferation of B9 cells as indicated by an increase in 3H-thymidine incorporation, CT-1 and LIF were inactive (Figure 7A), even at concentrations as high as 2 uM (data not shown). Thus, CT-1 does not mimic the activity of IL-6 in promoting B cell expansion.

While IL-6 stimulates the growth of several B cell lymphomas, myelomas, and plasmacytomas, it also has growth inhibitory effects on certain B lymphoma and myeloid leukemia cells (Akira *et al.*, *Adv. Immunol.*, 54:1-78 (1993)). IL-6 (as well as LIF and OSM) inhibits the growth of the mouse myeloid leukemia cell line, M1, and induces its differentiation into a macrophage-like phenotype (Akira *et al.*, *Adv. Immunol.*, 54:1-78 (1993); Rose *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8641-8645 (1991)). CT-1 was 6 fold more potent than LIF in inhibiting the uptake of 3H-thymidine by M1 cells (Fig. 7B). Thus, CT-1 does share at least some of the growth inhibitory activities of the IL-6 family cytokines .

Neuronal assays. Members of the IL-6 cytokine family modulate the phenotype and promote the survival of neuronal cells (Patterson, *Proc. Natl. Acad. Sci. USA*, 91:7833-7835 (1994)). LIF and CNTF can induce a switch in the transmitter phenotype of sympathetic neurons from noradrenergic to cholinergic, a change that is accompanied by the induction of several neuropeptides including substance P, somatostatin, and vasoactive intestinal polypeptide (Rao, *J. Neurobiol.*, 24:215-232 (1992)). The ability of CT-1 to induce this switch in the transmitter phenotype was determined with cultured rat sympathetic neurons. CT-1 inhibited the tyrosine hydroxylase activity (a noradrenergic marker) and stimulated somewhat the choline acetyltransferase activity (a cholinergic marker) of these cells, effects that paralleled the actions of LIF (Fig. 8A). Thus, CT-1 is active in modulating the phenotype of sympathetic neurons.

Parkinson's disease is caused by the degeneration of dopaminergic neurons of the midbrain (Hirsch *et al.*, *Nature*, 334:345-348 (1988)). While proteins of the neurotrophin family (brain-derived neurotrophic factor and neurotrophin-4/5) as well as of the TGF-β family (GDNF, TGF-β2 and TGF-β3) promote the survival of cultured dopaminergic neurons (Poulsen *et al.*, *Neuron*, 13:1245-1252 (1994)) many other growth factors and cytokines, including CNTF, do not. Unlike CNTF, CT-1 was found to promote the survival of rat dopaminergic neurons, although it was not as potent as GDNF (Fig. 8B).

While inactive on dopaminergic neurons, CNTF does promotes the survival of ciliary neurons (Ip *et al.*, *Prog. Growth Factor Res.*, 4:139-155 (1992)). CT-1 was tested for its activity in promoting the survival of chick ciliary neurons (Fig. 8C). While at maximal concentrations, CT-1 was as active as CNTF, the potency of CT-1 in promoting ciliary neuron survival was about 1000 fold less than that of CNTF (Fig. 8C). Thus, CT-1 shares some neuronal activities with the IL-6 family cytokines such as CNTF.

Embryonic development assay. The presence or absence of soluble factors plays a key role during embryonic and fetal development. For example, embryonic stem cells require the continuous presence of soluble factors secreted by fibroblasts to maintain their undifferentiated, pluripotent phenotype. LIF (Williams *et al.*, *Nature*, 336:688-690 (1988); Smith *et al.*, *Nature*, 336:688-690 (1988)), CNTF (Conover *et al.*, *Development*, 119:559-565 (1993)), and OSM (Rose *et al.*, *Cytokine*, 6:48-54 (1994))--but not IL-6 without the soluble IL-6 receptor (Yoshida *et al.*, *Mech. Dev.*, 45:163-171 (1994))--can replace these fibroblast-derived factors in

maintaining the pluripotent phenotype of embryonic stem cells in culture. CT-1 was also found to inhibit the differentiation of mouse embryonic stem cells (Fig. 9); it was as effective as LIF at the concentrations tested.

Thus, the data from seven *in vitro* biological assays indicate that CT-1 is active in assays where LIF is active and *vice versa*. Accordingly, these assays systems (and others in which CT-1 has a demonstrated activity as shown herein) can be used to screen for and identify CT-1 agonists and antagonists useful for treating disorders dependent upon or resultinf from the biological activity (or loss, reduction or overproducction of the activity) demonstrated in these assays. These data also show that CT-1 is active in assays where CNTF is active, but that the converse is not always the case, and that CT-1 is inactive in IL-6 specific assays, assays in which LIF is also inactive. Since the activity profiles of members of this cytokine family are determined by the receptors expressed on target cell populations, these data are consistent with the hypothesis that CT-1 binds and transduces its biological effects via the LIF receptor.

CT-1 binding to M1 cells. In order to show directly that CT-1 functions via the LIF receptor, binding was performed on M1 cells, where LIF binding has been previously characterized (Hilton *et al.; Proc. Natl. Acad. Sci. USA*, 85:5971-5975 (1988)). Both CT-1 and LIF inhibit the growth of this cell line (see above). Labeled CT-1 was specifically bound to M1 cells (Fig. 10A), and this binding was completely competed by unlabeled LIF (Fig. 10B). Similarly, labeled LIF binding was competed by both unlabeled LIF and CT-1 (Fig. 10C and 10D). These data suggest that CT-1 and LIF bind to the same receptor on M1 cells. Scatchard analysis yields a single class of binding sites in all cases: the binding parameters are similar regardless of the labeled ligand--K_d (CT-1] ~ 0.7 nM, K_d [LIF] ~ 0.2 nM, and ~ 1500 sites per cell.

Cross-linking of CT-1 on M1 cells. To analyze the protein(s) that bind CT-1 on the cell surface, labeled CT-1 and LIF were bound to M1 cells, chemically cross-linked, and the solubilized proteins analyzed by SDS gel electrophoresis (Fig. 11). Both ligands gave one specific band with a mobility of ~ 200 kDa, and in both cases this cross-linked band was competed by either unlabeled ligand. Thus, CT-1 and LIF interact with a protein of the same size on the surface of M1 cells; this protein has a mobility expected for the LIF receptor (Davis *et al.*, *Science*, 260:1805-1808 (1993); Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)).

Inhibition of CT-1 binding to M1 cells by an anti-gp130 monoclonal antibody. In order to show that gp130, the common signaling subunit shared by all receptors for ligands of the IL-6 cytokine family, is a part of the receptor binding complex for CT-1, the effect of an anti-gp130 monoclonal antibody on CT-1 binding was determined (Fig. 12A). This neutralizing antibody inhibited over 80 % of the specific CT-1 binding to M1 cells; no inhibition was found with comparable concentrations of a control antibody. These data indicate that gp130 is a component of the CT-1 receptor complex.

CT-1 induction of DNA binding activity in M1 cells. To show that CT-1 induces intracellular signaling events like those found for other cytokines that signal via gp130 (Yin *et al.*, *Exp. Hematol.*, 22:467-472 (1994);

Narazaki *et al.*, *Proc. Natl. Acad. USA*, 91:2285-2289 (1994); Zhong *et al.*, *Science*, 264:95-98 (1994); Akira *et al.*, *Cell*, 77:63-71 (1994)) DNA mobility shift assays wee performed with cell extracts from M1 cells (Fig. 12B). CT-1, like LIF, induced a shift in the mobility of the DNA element, SIE. Addition of the unlabeled element showed that the shifted band was specific. Thus, CT-1 induces the activation of a DNA binding activity like that expected for signaling via gp130 and activation of the Jak/STAT pathway.

CT-1 binding to cardiac myocytes. The binding of labeled CT-1 and LIF was also determined for rat cardiac myocytes, the cells used for the original assay and isolation of CT-1. Both ligands specifically bound and cross-competed for binding to these cells (Fig. 13A and 13B), as was the case for M1 cells. These data suggest that CT-1 and LIF bind and induce cardiac myocyte hypertrophy via the LIF receptor.

CT-1 binding to the soluble LIF receptor. In order to clarify whether CT-1 can bind directly to the LIF receptor or gp130 without the need for an additional membrane-bound component (as is the case for CNTF), binding experiments were performed with purified, soluble forms of the mouse LIF receptor and gp130 expressed as their extracellular domains containing a C-terminal histidine tag. Such experiments have recently shown that OSM binds directly to soluble gp130 ($K_d \sim 44$ nM for the human proteins) (Saadat *et al.*, *J. Cell Biol.*, 108:1807-1816 (1989)). On the other hand, LIF binds directly to the LIF binding protein, a naturally occurring soluble form of the LIF receptor ($K_d \sim 2$ nM for the mouse proteins) (Layton *et al.*, *J. Biol. Chem.*, 269:17048-17055 (1994); Layton *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:8616-8620 (1992)). The soluble mouse LIF receptor and gp130 were expressed in mammalian cells, purified by Ni^{++} chelate chromatography, and judged to be at least 90 % pure by SDS gel electrophoresis (data not shown). Binding experiments with labeled CT-1 show that it specifically binds to the soluble LIF receptor (Fig. 14A), as does labeled LIF (data not shown). CT-1 failed to bind to soluble gp130 at gp130 concentrations as high as 350 nM (Fig. 14B). The binding of CT-1 to the soluble LIF receptor was enhanced by the addition of soluble gp130 (Fig. 14C), suggesting that CT-1, soluble LIF receptor, and soluble gp130 form a tripartite complex as would be expected for the CT-1 activation of the LIF receptor complex. Competition binding experiments show that CT-1 binds to the soluble LIF receptor with a reasonable affinity, $K_d = 1.9$ nM (Fig. 14D). This affinity is about the same as that found for the binding of LIF ($K_d = 1.5$ nM, data not shown) and is the same as that found previously for LIF binding to the naturally occurring form of the soluble LIF receptor ($K_d = 1$-4 nM (48)). These data demonstrate that CT-1 interacts directly with the soluble LIF receptor without the need for an additional binding component. The results suggest that CT-1 (like LIF) binds first with a relatively low affinity to the LIF receptor on the cell membrane and then forms a heterotrimeric complex with a higher apparent affinity upon interaction with gp130.

DISCUSSION

In vitro hematopoietic, neuronal, and developmental assays have been used herein to show that CT-1 has a range of activities in addition to the induction of cardiac myocyte hypertrophy for which it was initially

isolated. As disclosed herein, CT-1 is more potent than LIF in inhibiting the growth of the myeloid leukemia cell line, M1; it induces a phenotypic switch in sympathetic neutrons; it promotes the survival of dopaminergic neurons from the central nervous system and ciliary neurons from the periphery; and it maintains the undifferentiated phenotype of embryonic stem cells. CT-1 and LIF share a common activity profile--both inhibit the growth of M1 cells, induce the switch in sympathetic neuron phenotype, inhibit the differentiation of embryonic stem cells, and induce cardiac myocyte hypertrophy. CT-1 is active in assays where CNTF is active--both induce the switch in sympathetic neuron phenotype (Saadat *et al.*, *J. Cell Biol.*, 108:1807-1816 (1989)) promote the survival of ciliary neurons, and inhibit the differentiation of embryonic stem cells (Conover *et al.*, *Development*, 119:559-565 (1993)). On the other hand, CT-1 is active in several assays where CNTF is inactive--inhibition of M1 cell growth (CNTF activity requires the inclusion of soluble CNTF receptor (Davis *et al.*, *Science*, 259:1736-1739 (1993)), promotion of dopaminergic neuron survival, and induction of cardiac myocyte hypertrophy. CT-1 is inactive, as are LIF and CNTF (Davis *et al.*, *Science*, 259:1736-1739 (1993); Kitamura *et al.*, *Trends Endo. Metabol.*, 5:87744-14 (1994)) in the stimulation B9 cell growth, an assay that is relatively specific for IL-6.

Alignments of the amino acid sequences of CT-1 and other members of the IL-6 cytokine family show that while these cytokines share biological activities and receptor subunits, they are only distantly related in primary sequence (14-24 % identity for the mammalian proteins, Fig. 15A). There is little conservation of the cysteine residues and only a partial maintenance of the exon-intron boundaries (Bruce *et al.*, *Prog. Growth Factor Res.*, 4:157-170 (1992); Bazan, *Neuron*, 7:197-208 (1991)). More sophisticated analyses (including the crystal structure of LIF (Robinson *et al.*, *Cell*, 77:1101-1116 (1994)) show that these proteins share a common structural architecture of four alpha helices (for reference see Bazan, *Neuron*, 7:197-208 (1991)). The individual family members are more related across species. The human and mouse sequences for CT-1, LIF, CNTF, or IL-11 are 79-88 % identical (Fig. 15A); the IL-6 homologues are 41 % identical. Some uncertainty remains as to whether the chick protein, identified as GPA, is the avian homologue of CNTF or another family member for which no mammalian homologue has yet been identified (Leung *et al.*, *Neuron*, 8:1045-1053 (1992); Richardson, *Pharmacol. Ther.*, 63:187-198 (1994)). CT-1 does not appear to be the mammalian homologue of GPA, as chicken GPA is more similar in amino sequence to mouse CNTF than to mouse CT-1 (46 verses 26 % identity, Fig. 15A). On the other hand, there are similarities among CT-1, CNTF, and GPA--all lack a conventional amino terminal, secretion signal sequence. Interestingly, CT-1 and GPA appear to be secreted from cells while CNTF is not (Leung *et al.*, *Neuron*, 8:1045-1053 (1992); Stockli *et al.*, *Nature*, 342:920-923 (1989); Lin *et al.*, *Science*, 246:1023-1025 (1989)).

As is shown diagrammatically in Fig. 15B, the receptors for cytokines of the IL-6 family are composed of related subunits some of which are cytokine specific and some of which are shared (Davis *et al.*, *Curr. Opin.*

Cell Biol., 5:281-285 (1993); Stahl *et al.*, *Cell*, 74:587-590 (1993); Kishimoto *et al.*, *Cell*, 76:253-262 (1994); Hilton et al., *EMBO J.*, 13:4765-4775 (1994)). All the receptors in this family have in common the transmembrane signaling subunit, gp130. The binding of IL-6 to the 80 kDa IL-6 receptor α subunit leads to the dimerization of gp130 as the first step in signal transduction. Similarly, the binding of IL-11 to the IL-11 receptor also leads to gp130 dimerization. LIF, OSM, and CNTF induce the heterodimeriztion of gp130 and with another signaling subunit, the LIF receptor. LIF and OSM bind directly to the LIF receptor or gp130 and induce dimerization without a ligand-specific α subunit, while CNTF binds first to the GPI-linked CNTF receptor. While the formation of receptor complexes containing homo- or heterodimers of gp130 is believed to be an essential signaling event, the exact stoichiometry of the subunits in the complex is not known in most cases. For the IL-6 receptor, a recent report concludes that the signaling complex is a hexamer containing two 20 kDa ligands, two 80 kDa IL-6 receptors, and two 130 kDa gp130 molecules (Ward *et al.*, *J. Biol. Chem.*, 269:23286-23289 (1994)). The ligand-induced dimerization of gp130 or gp130 and LIF receptor leads to the tyrosine phosphorylation and activation of associated tyrosine kinases of the Jak family (Jak1, Jak2, and Tyk2) followed by the activation of transcription factors of the STAT family (STAT1 and STAT3) (Lütticken *et al.*, *Science*, 263:89-92 (1994); Stahl *et al.*, *Science*, 263:92-95 (1994); Yin *et al.*, *Exp. Hematol.*, 22:467-472 (1994); Narazaki *et al.*, *Proc. Natl. Acad. USA*, 91:2285-2289 (1994); Zhong *et al.*, *Science*, 264:95-98 (1994); Akira *et al.*, *Cell*, 77:63-71 (1994)). Although not meant to be limiting, it is proposed that the activation of the Jak-STAT pathway is probably one of the key steps in the signal transduction mechanism for most if not all the actions of the IL-6 family cytokines, including CT-1.

The presence or absence of the different subunits of the IL-6 family receptors dictates the responsiveness of various cells to the different cytokines (Taga et al., *FASEB J.* 6:3387-3396 (1992); Kishimoto *et al.*, *Cell*, 76:253-262 (1994)). Thus, all responsive cells are believed to express gp130. B9 cells fail to respond to LIF and CNTF because they lack LIF receptor, IL-6 is inactive on embryonic stem cells because these cells lack the IL-6 receptor α subunit, LIF is active on M1 cells because both gp130 and LIF receptor are present, while CNTF is inactive due to a lack of CNTF receptor α, etc. Based on the profile of CT-1 activities reported here, CT-1 functions via the LIF receptor. This is established directly herein as follows. First, as shown herein, CT-1 and LIF completely cross-compete for binding to M1 cells, a cell line where LIF binding has been previously well characterized, K_d [LIF] = 0.1-0.2 nM (Hilton *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:5971-5975 (1988); Gearing *et al.*, *New Biologist*, 4:61-65 (1992)). Regardless of which ligand is used as the label or competitor, an affinity for CT-1, $K_d \sim 0.7$ nM which is 3-4 fold less than that found for LIF, $K_d \sim 0.2$ nM is found. Secondly, cross-linking data show that CT-1 and LIF specifically interact with a protein of ~ 200 kDa, a protein about the size expected for the LIF receptor (Davis *et al.*, *Science*, 260:1805-1808 (1993); Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)). Third, as shown herein, an anti-gp130 monoclonal antibody specifically

inhibits the binding of labeled CT-1 to M1 cells, showing that gp130 is a component of the CT-1 receptor complex. Fourth, CT-1 induces the activation of a DNA binding activity, an intracellular signaling event induced by LIF and other members of the IL-6 cytokine family in the course of activation of the Jak/STAT pathway (Lütticken *et al.*, *Science*, 263:89-92 (1994); Yin *et al.*, *Exp. Hematol.*, 22:467-472 (1994); Zhong *et al.*, *Science*, 264:95-98 (1994); Akira *et al.*, *Cell*, 77:63-71 (1994)). These data demonstrate that CT-1 can bind to and activate the LIF receptor complex. This finding does not exclude the possibility that some cells have an additional CT-1 specific receptor or receptor subunit that forms a heterodimer with gp130, as has been reported for OSM (Mosley *et al.*, *Cytokine*, 6:554 (1994)).

As shown herein, CT-1 and LIF also cross-compete for binding to rat cardiac myocytes. This finding is consistent with the hypothesis that these two ligands act on these cells via the LIF receptor, as established herein for M1 cells.

While LIF and OSM induce the heterodimerization of the same receptor subunits, LIF receptor and gp130, the affinity of these two ligands for the individual receptor components differs. LIF binds to the LIF receptor (K_d ~ 2 nM (Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)) but does not interact with gp130 in the absence of the LIF receptor. Conversely, OSM binds to gp130 (K_d ~ 1 nM (Liu *et al.*, *J. Biol. Chem.*, 267:16763-16766 (1992)) but does not bind to the LIF receptor alone (Gearing *et al.*, *EMBO J.*, 10:2839-2848 (1991)). Soluble forms of these two receptor subunits, consisting of their extracellular domains, are found in the circulation (Layton *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:8616-8620 (1992); Narazaki *et al.*, *Blood*, 82:1120-1126 (1993)). The soluble LIF binding protein binds LIF with a K_d ~ 2 nM (for the mouse proteins) (Layton *et al.*, *J. Biol. Chem.*, 269:17048-17055 (1994)), while a recombinant form of soluble gp130 binds OSM with a K_d ~ 44 nM (for the human proteins) (Sporeno *et al.*, *J. Biol. Chem.*, 269:10991-10995 (1994)). As shown herein, CT-1 binds to the soluble LIF receptor with about the same affinity as LIF (K_d ~ 2 nM, for the mouse proteins) and in the absence of other proteins. CT-1 does not bind to soluble mouse gp130 even at high concentrations. The addition of soluble gp130 does increase the binding of CT-1 to the soluble LIF receptor, however, presumably by the formation of a heterotrimeric complex. The concentration of soluble gp130 required for this effect (~ 100 nM), while high by solution binding standards, is readily attainable on the surface of a cell. For example, 500 molecules of gp130 expressed on the surface of a cell of 10 μm diameter would have an effective concentration of ~ 300 nM in a 100 Å shell surrounding the cell, see (Ward *et al.*, *J. Biol. Chem.*, 269:23286-23289 (1994)). Thus, these results indicate that CT-1 binds to the LIF receptor in the same manner as LIF, by first binding with low affinity to the LIF receptor subunit, an interaction that does not require additional components, and second by recruiting gp130 to form a high affinity signaling complex. Although CT-1 was isolated based on its ability to induce cardiac myocyte hypertrophy, it clearly has a much wider range of activities, as is found for the other cytokines of the IL-6 family (Kishimoto *et al.*, *Science*, 258:593-597 (1992);

Kishimoto *et al.*, *Cell*, 76:253-262 (1994)). The receptor data presented here predict that CT-1 will mimic the many effects of LIF *in vitro* and *in vivo*. Some of the functions of LIF, and thus targets for CT-1 and its antagonists or agonists, are obtained from the targeted deletion of the LIF gene in mice, which leads to animals that are outwardly normal although they do exhibit a reduced growth rate, a decrease in hematopoietic cells, and a failure of proper embryo implantation (Escary *et al.*, *Nature*, 263:361-364 (1993)). These studies are consistent with the *in vitro* data presented herein and the uses of CT-1 and its antagonists and agonists.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the description herein and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.
The Regents of the University of California

(ii) TITLE OF INVENTION: Cardiotrophin and Uses Therefor

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Torchia, PhD., Timothy E.
(B) REGISTRATION NUMBER: 36,700
(C) REFERENCE/DOCKET NUMBER: P0994PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-8674
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1352 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATAAGCCT GGGGCCAGCA TGAGCCAGAG GGAGGGAAGT CTGGAAGACC 50

ACCAGACTGA CTCCTCAATC TCATTCCTAC CCCATTTGGA GGCCAAGATC 100

CGCCAGACAC ACAACCTTGC CCGCCTCCTG ACCAAATATG CAGAACAACT 150

TCTGGAGGAA TACGTGCAGC AACAGGGAGA GCCCTTTGGG CTGCCGGGCT 200

TCTCACCACC GCGGCTGCCG CTGGCCGGCC TGAGTGGCCC GGCTCCGAGC 250

CATGCAGGGC TACCGGTGTC CGAGCGGCTG CGGCAGGATG CAGCCGCCCT 300

GAGTGTGCTG CCCGCGCTGT TGGATGCCGT CCGCCGCCGC CAGGCGGAGC 350

TGAACCCGCG CGCCCCGCGC CTGCTGCGGA GCCTGGAGGA CGCAGCCCGC 400

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GGGCCAGCTG GTGCCAGGGG GCGTCGCCTG AGAGTGAATA CTTTTTCTTG 650

TAAGCTCGCT CTGTCTCGCC TCTTTGGCTT CAAATTTTCT GTCTCTCCAT 700

CTGTGTCCTG TGTGTTCTTG GGCTGTCCCT ATCTTTCTGC ATTTGTGTGG 750

TCTCTCTCTT CTGCTCTCCT CTCTGCAGGG AGCTTCTTTT TTCCAACAGT 800

TTCTCGTTTT GTCTCTCTCC AGTCTTGAAC ACTTTTGTCT CCGAGAGGTC 850

TCTTTTTGTT TCCTTGTCTC TTGGTTCTTT CTTTGCTTGC TTGCTTGCTT 900

GCTTGCTTGT TGTTGAGACA GGGTCTCACC ATATAGCTCT GGATGGCCTG 950

GAACTTGCTA TGTAGGCCAG GCTGGCCTCC AGCTCATAGA GATCCACTTG 1000

CCTCCGACTC CCAATTTCCC CATCTGTCTC CCTGTGATCC ATATGGGTAT 1050

GTGTAACCCT TACTTTGTCT CATGGAGGTG ACAATTTTTC TCCCTTCAGT 1100

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AGGCCATTCA CCTTAGGACC TTCCCACCAG TTCCTTTGTA GGCAAATCCC 1200

TCCCCCTTTG AGGTCCTTCC CTTTCATACC GCCCTAGGCT GGTCAATGGA 1250

GAGAGAAAGG CAGAAAAACA TCTTTAAAGA GTTTTATTTG AGAATAAATT 1300

AATTTTTGTA AATAAAATGT TTAACAATAA AACTAAACTT TTATGAAAAA 1350

AA 1352

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1352 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTATTCGGA CCCCGGTCGT ACTCGGTCTC CCTCCCTTCA GACCTTCTGG 50

TGGTCTGACT GAGGAGTTAG AGTAAGGATG GGGTAAACCT CCGGTTCTAG 100

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AGACCTCCTT ATGCACGTCG TTGTCCCTCT CGGGAAACCC GACGGCCCGA 200

AGAGTGGTGG CGCCGACGGC GACCGGCCGG ACTCACCGGG CCGAGGCTCG 250

GTACGTCCCG ATGGCCACAG GCTCGCCGAC GCCGTCCTAC GTCGGCGGGA 300

CTCACACGAC GGGCGCGACA ACCTACGGCA GGCGGCGGCG GTCCGCCTCG 350

ACTTGGGCGC GCGGGGCGCG GACGACGCCT CGGACCTCCT GCGTCGGGCG 400

GTCCAAGCCC GGGACCCGCG GCGCCACCTC TGTCACGACC GGCGCGACCC 450

GCGACGTCGG GCGCCCGGGC CCGGTCTCGG GCAGTGGCAG CGGTGGGAGA 500

AGTGCCGGTT GTCGTGACGT CCGTAGAAGA GTCGGTTCCA CGACCCCAAG 550

GTGCACACGC CGGAGATACC GCTCACCCAC TCGGCGTGTC TCCCGCTGGA 600

CCCGGTCGAC CACGGTCCCC CGCAGCGGAC TCTCACTTAT GAAAAAGAAC 650

ATTCGAGCGA GACAGAGCGG AGAAACCGAA GTTTAAAAGA CAGAGAGGTA 700

GACACAGGAC ACACAAGAAC CCGACAGGGA TAGAAAGACG TAAACACACC 750

AGAGAGAGAA GACGAGAGGA GAGACGTCCC TCGAAGAAAA AAGGTTGTCA 800

AAGAGCAAAA CAGAGAGAGG TCAGAACTTG TGAAAACAGA GGCTCTCCAG 850

AGAAAAACAA AGGAACAGAG AACCAAGAAA GAAACGAACG AACGAACGAA 900

CGAACGAACA ACAACTCTGT CCCAGAGTGG TATATCGAGA CCTACCGGAC 950

CTTGAACGAT ACATCCGGTC CGACCGGAGG TCGAGTATCT CTAGGTGAAC 1000

GGAGGCTGAG GGTTAAAGGG GTAGACAGAG GGACACTAGG TATACCCATA 1050

CACATTGGGA ATGAAACAGA GTACCTCCAC TGTTAAAAAG AGGGAAGTCA 1100

AAGAAACAAG AAATGACTGG TCTTTTCACG GATGAACAGG GGACCACCGT 1150

TCCGGTAAGT GGAATCCTGG AAGGGTGGTC AAGGAAACAT CCGTTTAGGG 1200

AGGGGGAAAC TCCAGGAAGG GAAAGTATGG CGGGATCCGA CCAGTTACCT 1250

CTCTCTTTCC GTCTTTTTGT AGAAATTTCT CAAAATAAAC TCTTATTTAA 1300

TTAAAAACAT TTATTTTACA AATTGTTATT TTGATTTGAA AATACTTTTT 1350

TT 1352

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 203 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Gln Arg Glu Gly Ser Leu Glu Asp His Gln Thr Asp Ser
1 5 10 15

Ser Ile Ser Phe Leu Pro His Leu Glu Ala Lys Ile Arg Gln Thr
20 25 30

His Asn Leu Ala Arg Leu Leu Thr Lys Tyr Ala Glu Gln Leu Leu
35 40 45

Glu Glu Tyr Val Gln Gln Gln Gly Glu Pro Phe Gly Leu Pro Gly
50 55 60

Phe Ser Pro Pro Arg Leu Pro Leu Ala Gly Leu Ser Gly Pro Ala
65 70 75

Pro Ser His Ala Gly Leu Pro Val Ser Glu Arg Leu Arg Gln Asp
80 85 90

Ala Ala Ala Leu Ser Val Leu Pro Ala Leu Leu Asp Ala Val Arg
95 100 105

Arg Arg Gln Ala Glu Leu Asn Pro Arg Ala Pro Arg Leu Leu Arg
110 115 120

Ser Leu Glu Asp Ala Ala Arg Gln Val Arg Ala Leu Gly Ala Ala
125 130 135

Val Glu Thr Val Leu Ala Ala Leu Gly Ala Ala Ala Arg Gly Pro
140 145 150

Gly Pro Glu Pro Val Thr Val Ala Thr Leu Phe Thr Ala Asn Ser
155 160 165

Thr Ala Gly Ile Phe Ser Ala Lys Val Leu Gly Phe His Val Cys
170 175 180

Gly Leu Tyr Gly Glu Trp Val Ser Arg Thr Glu Gly Asp Leu Gly
185 190 195

Gln Leu Val Pro Gly Gly Val Ala
200 203

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Phe Thr Glu His Ser Pro Leu Thr Pro His Arg Arg Asp
1 5 10 15

Leu Cys Ser Arg Ser Ile Trp Leu Ala Arg Lys Ile Arg Ser Asp
20 25 30

Leu Thr Ala Leu Thr Glu Ser Tyr Val Lys His Gln Gly Leu Asn
35 40 45

Lys Asn Ile Asn Leu Asp Ser Ala Asp Gly Met Pro Val Ala Ser
50 55 60

Thr Asp Gln Trp Ser Glu Leu Thr Glu Ala Glu Arg Leu Gln Glu
65 70 75

Asn Leu Gln Ala Tyr Arg Thr Phe His Val Leu Leu Ala Arg Leu

80 85 90

Leu Glu Asp Gln Gln Val His Phe Thr Pro Thr Glu Gly Asp Phe
95 100 105

His Gln Ala Ile His Thr Leu Leu Leu Gln Val Ala Ala Phe Ala
110 115 120

Tyr Gln Ile Glu Glu Leu Met Ile Leu Leu Glu Tyr Lys Ile Pro
125 130 135

Arg Asn Glu Ala Asp Gly Met Pro Ile Asn Val Gly Asp Gly Gly
140 145 150

Leu Phe Glu Lys Lys Leu Trp Gly Leu Lys Val Leu Gln Glu Leu
155 160 165

Ser Gln Trp Thr Val Arg Ser Ile His Asp Leu Arg Phe Ile Ser
170 175 180

Ser His Gln Thr Gly Ile Pro Ala Arg Gly Ser His Tyr Ile Ala
185 190 195

Asn Asn Lys Lys Met
200

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGGCCGCGA GCTCGAATTC TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT 50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1018 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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ACGCTGAGCA GCTGCTCCAG GAATATGTGC AGCTCCAGGG AGACCCCTTC 200

GGGCTGCCCA GCTTCTCGCC GCCGCGGCTG CCGGTGGCCG GCCTGAGCGC 250

CCCGGCTCCG AGCCACGCGG GGCTGCCAGT GCACGAGCGG CTGCGGCTGG 300

ACGCGGCGGC GCTGGCCGCG CTGCCCCCGC TGCTGGACGC AGTGTGTCGC 350

CGCCAGGCCG AGCTGAACCC GCGCGCGCCG CGCCTGCTGC GCCGCCTGGA 400

GGACGCGGCG CGCCAGGCCC GGGCCCTGGG CGCCGCCGTG GAGGCCTTGC 450

TGGCCGCGCT GGGCGCCGCC AACCGCGGGC CCCGGGCCGA GCCCCCCGCC 500

GCCACCGCCT CAGCCGCCTC CGCCACCGGG GTCTTCCCCG CCAAGGTGCT 550

GGGGCTCCGC GTTTGCGGCC TCTACCGCGA GTGGCTGAGC CGCACCGAGG 600

GCGACCTGGG CCAGCTGCTG CCCGGGGGCT CGGCCTGAGC GCCGCGGGGC 650

AGCTCGCCCC GCCTCCTCCC GCTGGGTTCC GTCTCTCCTT CCGCTTCTTT 700

GTCTTTCTCT GCCGCTGTCG GTGTCTGTCT GTCTGCTCTT AGCTGTCTCC 750

ATTGCCTCGG CCTTCTTTGC TTTTTGTGGG GGAGAGGGGA GGGGACGGGC 800

AGGGTCTCTG TCGCCCAGGC TGGGGTGCAG TGGCGCGATC CCAGCACTGC 850

AGCCTCAACC TCCTGGGCTC AAGCCATCCT TCCGCCTCAG CTTCCCCAGC 900

AGCTGGGACT ACAGGCACGC GCCACCACAG CCGGCTAATT TTTTATTTAA 950

TTTTTTGTAG AGACGAGGTT TCGCCATGTT GCCCAGGCTG GTCTTGAACT 1000

CCGGGGCTCA AGCGATCC 1018

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1018 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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TCAGACCTTC TGGGGGTCTG ACTAAGGAGT CAGAGTGAAG AAGGGGTGAA 100

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CCCGACGGGT CGAAGAGCGG CGGCGCCGAC GGCCACCGGC CGGACTCGCG 250

GGGCCGAGGC TCGGTGCGCC CCGACGGTCA CGTGCTCGCC GACGCCGACC 300

TGCGCCGCCG CGACCGGCGC GACGGGGGCG ACGACCTGCG TCACACAGCG 350

GCGGTCCGGC TCGACTTGGG CGCGCGCGGC GCGGACGACG CGGCGGACCT 400

CCTGCGCCGC GCGGTCCGGG CCCGGGACCC GCGGCGGCAC CTCCGGAACG 450

ACCGGCGCGA CCCGCGGCGG TTGGCGCCCG GGGCCCGGCT CGGGGGGCGG 500

CGGTGGCGGA GTCGGCGGAG GCGGTGGCCC CAGAAGGGGC GGTTCCACGA 550

CCCCGAGGCG CAAACGCCGG AGATGGCGCT CACCGACTCG GCGTGGCTCC 600

CGCTGGACCC GGTCGACGAC GGGCCCCCGA GCCGGACTCG CGGCGCCCCG 650

TCGAGCGGGG CGGAGGAGGG CGACCCAAGG CAGAGAGGAA GGCGAAGAAA 700

CAGAAAGAGA CGGCGACAGC CACAGACAGA CAGACGAGAA TCGACAGAGG 750

TAACGGAGCC GGAAGAAACG AAAAACACCC CCTCTCCCCT CCCCTGCCCG 800

TCCCAGAGAC AGCGGGTCCG ACCCCACGTC ACCGCGCTAG GGTCGTGACG 850

TCGGAGTTGG AGGACCCGAG TTCGGTAGGA AGGCGGAGTC GAAGGGGTCG 900

TCGACCCTGA TGTCCGTGCG CGGTGGTGTC GGCCGATTAA AAAATAAATT 950

AAAAAACATC TCTGCTCCAA AGCGGTACAA CGGGTCCGAC CAGAACTTGA 1000

GGCCCCGAGT TCGCTAGG 1018

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 amino acids
(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Ser Arg Arg Glu Gly Ser Leu Glu Asp Pro Gln Thr Asp Ser
1 5 10 15

Ser Val Ser Leu Leu Pro His Leu Glu Ala Lys Ile Arg Gln Thr
20 25 30

His Ser Leu Ala His Leu Leu Thr Lys Tyr Ala Glu Gln Leu Leu
35 40 45

Gln Glu Tyr Val Gln Leu Gln Gly Asp Pro Phe Gly Leu Pro Ser
50 55 60

Phe Ser Pro Pro Arg Leu Pro Val Ala Gly Leu Ser Ala Pro Ala
65 70 75

Pro Ser His Ala Gly Leu Pro Val His Glu Arg Leu Arg Leu Asp
80 85 90

Ala Ala Ala Leu Ala Ala Leu Pro Pro Leu Leu Asp Ala Val Cys
95 100 105

Arg Arg Gln Ala Glu Leu Asn Pro Arg Ala Pro Arg Leu Leu Arg
110 115 120

Arg Leu Glu Asp Ala Ala Arg Gln Ala Arg Ala Leu Gly Ala Ala
125 130 135

Val Glu Ala Leu Leu Ala Ala Leu Gly Ala Ala Asn Arg Gly Pro
140 145 150

Arg Ala Glu Pro Pro Ala Ala Thr Ala Ser Ala Ala Ser Ala Thr
155 160 165

Gly Val Phe Pro Ala Lys Val Leu Gly Leu Arg Val Cys Gly Leu
170 175 180

Tyr Arg Glu Trp Leu Ser Arg Thr Glu Gly Asp Leu Gly Gln Leu
185 190 195

Leu Pro Gly Gly Ser Ala
200 201

WHAT IS CLAIMED IS:

1. A method of enhancing the maintenance of pregnancy in a mammal into which an embryo has been introduced, the method comprising prior to said introducing, culturing at least one embryo in a medium containing an amount of CT-1 for sufficient time and under appropriate conditions so as to effect an enhancement of the maintenance of pregnancy in said mammal.

2. The method according to claim 1, wherein said mammal is selected from the group consisting of human, sheep, pig, cow, goat, donkey, horse, dog and cat.

3. The method according to claim 1, wherein CT-1 is of human or murine origin.

4. The method according to claim 1, wherein the medium for maintenance of the embryo is SOF or M2 medium.

5. A composition, comprising pluripotential embryonic stem cells and CT-1, a fibroblast growth factor, membrane associated steel factor, and soluble steel factor, the factors present in amounts to enhance the growth of and allow the continued proliferation of the cells.

6. A composition, comprising primordial germ cells and CT-1, a fibroblast growth factor, membrane associated steel factor and soluble steel factor, the factors present in amounts to enhance the growth of and allow the continued proliferation of the cells.

7. A composition, comprising embryonic ectoderm cells and CT-1, fibroblast growth factor, membrane associated steel factor and soluble steel factor, the factors present in amounts to enhance the growth of and allow the continued proliferation of the cells.

8. A composition, comprising CT-1, fibroblast growth factor, membrane associated steel factor, and soluble steel factor in amounts to enhance the growth of and allow the continued proliferation of primordial germ cells.

9. A composition, comprising CT-1, a fibroblast growth factor, membrane associated steel factor, and soluble steel factor in amounts to promote the formation of pluripotent embryonic stem cells from primordial germ cells.

10. A method of making a mammalian pluripotential embryonic stem cell, comprising administering a growth enhancing amount of CT-1, a basic fibroblast growth factor, membrane associated steel factor, and soluble steel factor to primordial germ cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

11. A method of making a pluripotential embryonic stem cell comprising administering a growth enhancing amount of CT-1, a basic fibroblast growth factor, membrane associated steel factor, and soluble steel factor to embryonic ectoderm cells under cell growth conditions, thereby making a pluripotential embryonic stem cell.

12. A method of stimulating the proliferation and differentiation of mammalian satellite cells into myoblasts, which method comprises contacting said cells with a stimulation-effective amount of CT-1 for a time and under conditions sufficient for said satellite cells to proliferate and differentiate into myoblasts.

13. The method according to claim 1 which further comprises the addition of one or more other cytokines in simultaneous or sequential combination with CT-1.

14. A method of myoblast transfer, comprising contacting mammalian satellite cells with a proliferation- and differentiation-effective amount of CT-1 for a time and under conditions sufficient for said satellite cells to proliferate and differentiate into myoblasts and then administering said myoblasts at multiple sites into muscles.

15. A method of treating a neoplastic disorder, comprising administering to a population of cells that comprise neoplastic cells of a patient in need of such treatment a therapeutically effective amount of CT-1.

16. The method of claim 16, wherein the neoplastic disorder is selected from the group consisting of a carcinoma, sarcoma, melanoma, lymphoma, and leukemia.

17. The method of claim 16, wherein the neoplastic cells are *in vitro*.

18. The method of claim 17, wherein the CT-1 is administered bone marrow to eliminate malignant cells from marrow for autologous marrow transplants.

19. A method of treating a mammal afflicted with arthritis or an inflammatory disease, comprising administering to the mammal in need of such treatment an amount of CT-1 antagonist which is effective for alleviation of the condition.

20. A method of treating a neuron other than a ciliary ganglion neuron, comprising providing the neuron with an amount of CT-1 effective to promote neuronal survival, growth, regeneration, or sprouting.

21. The method of claim 20, wherein the neuron is *in vivo*.

22. The method of claim 20, wherein the neuron is a central nervous system neuron.

23. A method of modulating a neuron's phenotype, comprising providing the neuron with an amount of CT-1 effective to promote a change in neuronal phenotype.

24. The method of claim 23, wherein the change is in the transmitter phenotype of the neuron.

25. The method of claim 23, wherein the neuron is *in vivo*.

```
   1 GGATAAGCCT GGGGCCAGCA TGAGCCAGAG GGAGGGAAGT CTGGAAGACC
     CCTATTCGGA CCCCGGTCGT ACTCGGTCTC CCTCCCTTCA GACCTTCTGG
   1                       M   S  Q  R   E  G  S   L  E  D  H

  51 ACCAGACTGA CTCCTCAATC TCATTCCTAC CCCATTTGGA GGCCAAGATC
     TGGTCTGACT GAGGAGTTAG AGTAAGGATG GGGTAAACCT CCGGTTCTAG
  12   Q  T  D   S  S  I   S  F  L  P   H  L  E   A  K  I

 101 CGCCAGACAC ACAACCTTGC CCGCCTCCTG ACCAAATATG CAGAACAACT
     GCGGTCTGTG TGTTGGAACG GGCGGAGGAC TGGTTTATAC GTCTTGTTGA
  28 R  Q  T  H   N  L  A   R  L  L   T  K  Y  A   E  Q  L

 151 TCTGGAGGAA TACGTGCAGC AACAGGGAGA GCCCTTTGGG CTGCCGGGCT
     AGACCTCCTT ATGCACGTCG TTGTCCCTCT CGGGAAACCC GACGGCCCGA
  45  L  E  E   Y  V  Q  Q   Q  G  E   P  F  G   L  P  G  F

 201 TCTCACCACC GCGGCTGCCG CTGGCCGGCC TGAGTGGCCC GGCTCCGAGC
     AGAGTGGTGG CGCCGACGGC GACCGGCCGG ACTCACCGGG CCGAGGCTCG
  62   S  P  P   R  L  P   L  A  G  L   S  G  P   A  P  S

 251 CATGCAGGGC TACCGGTGTC CGAGCGGCTG CGGCAGGATG CAGCCGCCCT
     GTACGTCCCG ATGGCCACAG GCTCGCCGAC GCCGTCCTAC GTCGGCGGGA
  78 H  A  G  L   P  V  S   E  R  L   R  Q  D  A   A  A  L

 301 GAGTGTGCTG CCCGCGCTGT TGGATGCCGT CCGCCGCCGC CAGGCGGAGC
     CTCACACGAC GGGCGCGACA ACCTACGGCA GGCGGCGGCG GTCCGCCTCG
  95  S  V  L   P  A  L  L   D  A  V   R  R  R   Q  A  E  L

 351 TGAACCCGCG CGCCCCGCGC CTGCTGCGGA GCCTGGAGGA CGCAGCCCGC
     ACTTGGGCGC GCGGGGCGCG GACGACGCCT CGGACCTCCT GCGTCGGGCG
 112   N  P  R   A  P  R   L  L  R  S   L  E  D   A  A  R

 401 CAGGTTCGGG CCCTGGGCGC CGCGGTGGAG ACAGTGCTGG CCGCGCTGGG
     GTCCAAGCCC GGGACCCGCG GCGCCACCTC TGTCACGACC GGCGCGACCC
 128 Q  V  R  A   L  G  A   A  V  E   T  V  L  A   A  L  G

 451 CGCTGCAGCC CGCGGGCCCG GGCCAGAGCC CGTCACCGTC GCCACCCTCT
     GCGACGTCGG GCGCCCGGGC CCGGTCTCGG GCAGTGGCAG CGGTGGGAGA
 145  A  A  A   R  G  P  G   P  E  P   V  T  V   A  T  L  F

 501 TCACGGCCAA CAGCACTGCA GGCATCTTCT CAGCCAAGGT GCTGGGGTTC
     AGTGCCGGTT GTCGTGACGT CCGTAGAAGA GTCGGTTCCA CGACCCCAAG
 162   T  A  N   S  T  A   G  I  F  S   A  K  V   L  G  F

 551 CACGTGTGCG GCCTCTATGG CGAGTGGGTG AGCCGCACAG AGGGCGACCT
     GTGCACACGC CGGAGATACC GCTCACCCAC TCGGCGTGTC TCCCGCTGGA
 178 H  V  C  G   L  Y  G   E  W  V   S  R  T  E   G  D  L

 601 GGGCCAGCTG GTGCCAGGGG GCGTCGCCTG AGAGTGAATA CTTTTTCTTG
     CCCGGTCGAC CACGGTCCCC CGCAGCGGAC TCTCACTTAT GAAAAAGAAC
 195  G  Q  L   V  P  G  G   V  A  O
```

FIG._1A

```
 651 TAAGCTCGCT CTGTCTCGCC TCTTTGGCTT CAAATTTTCT GTCTCTCCAT
     ATTCGAGCGA GACAGAGCGG AGAAACCGAA GTTTAAAAGA CAGAGAGGTA

 701 CTGTGTCCTG TGTGTTCTTG GGCTGTCCCT ATCTTTCTGC ATTTGTGTGG
     GACACAGGAC ACACAAGAAC CCGACAGGGA TAGAAAGACG TAAACACACC

 751 TCTCTCTCTT CTGCTCTCCT CTCTGCAGGG AGCTTCTTTT TTCCAACAGT
     AGAGAGAGAA GACGAGAGGA GAGACGTCCC TCGAAGAAAA AAGGTTGTCA

 801 TTCTCGTTTT GTCTCTCTCC AGTCTTGAAC ACTTTTGTCT CCGAGAGGTC
     AAGAGCAAAA CAGAGAGAGG TCAGAACTTG TGAAAACAGA GGCTCTCCAG

 851 TCTTTTTGTT TCCTTGTCTC TTGGTTCTTT CTTTGCTTGC TTGCTTGCTT
     AGAAAAACAA AGGAACAGAG AACCAAGAAA GAAACGAACG AACGAACGAA

 901 GCTTGCTTGT TGTTGAGACA GGGTCTCACC ATATAGCTCT GGATGGCCTG
     CGAACGAACA ACAACTCTGT CCCAGAGTGG TATATCGAGA CCTACCGGAC

 951 GAACTTGCTA TGTAGGCCAG GCTGGCCTCC AGCTCATAGA GATCCACTTG
     CTTGAACGAT ACATCCGGTC CGACCGGAGG TCGAGTATCT CTAGGTGAAC

1001 CCTCCGACTC CCAATTTCCC CATCTGTCTC CCTGTGATCC ATATGGGTAT
     GGAGGCTGAG GGTTAAAGGG GTAGACAGAG GGACACTAGG TATACCCATA

1051 GTGTAACCCT TACTTTGTCT CATGGAGGTG ACAATTTTTC TCCCTTCAGT
     CACATTGGGA ATGAAACAGA GTACCTCCAC TGTTAAAAAG AGGGAAGTCA

1101 TTCTTTGTTC TTTACTGACC AGAAAAGTGC CTACTTGTCC CCTGGTGGCA
     AAGAAACAAG AAATGACTGG TCTTTTCACG GATGAACAGG GGACCACCGT

1151 AGGCCATTCA CCTTAGGACC TTCCCACCAG TTCCTTTGTA GGCAAATCCC
     TCCGGTAAGT GGAATCCTGG AAGGGTGGTC AAGGAAACAT CCGTTTAGGG

1201 TCCCCCTTTG AGGTCCTTCC CTTTCATACC GCCCTAGGCT GGTCAATGGA
     AGGGGGAAAC TCCAGGAAGG GAAAGTATGG CGGGATCCGA CCAGTTACCT

1251 GAGAGAAAGG CAGAAAAACA TCTTTAAAGA GTTTTATTTG AGAATAAATT
     CTCTCTTTCC GTCTTTTTGT AGAAATTTCT CAAAATAAAC TCTTATTTAA

1301 AATTTTTGTA AATAAAATGT TTAACAATAA AACTAAACTT TTATGAAAAA
     TTAAAAACAT TTATTTTACA AATTGTTATT TTGATTTGAA AATACTTTTT

1351 AA (polyA)
     TT
```

FIG._1B

```
                  10         20         30         40         50
chf.781   MSQREGSLEDHQTDSSISFLPHLEAKIRQTHNLARLLTKYAEQLLEEYVQ
                             **         ***          * * **
humcntf             MAFTEHSPLTPHRRDLCSRSIWLARKIRSDLTALTESYVK
                            10         20         30         40

                  60         70         80         90        100
chf.781   QQGEPFGLPGFSPPRLPLAGLSGPAPSHAGLPVSERLRQDAAALSVLPAL
           **         *    * *                ***      *       *
humcntf   HQGLNKNINLDSADGMPVA----STDQWSELTEAERLQENLQAYRTFHVL
                  50              60         70         80

                 110        120        130        140
chf.781   LD-AVRRRQAELNPRAPRLLRSLEDAARQVRALGAAVETVLAALGAAARG
          *      *   *                  **  *     *     *
humcntf   LARLLEDQQVHFTPTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPR
            90        100        110        120        130

        150        160        170        180        190
chf.781   PGPEPVTVATLFTANSTAGIFSAKVLGFHVCGLYGEWVSRTEGDLGQLVP
                             * * * * *      * * **
humcntf   NEADGMPINV-----GDGGLFEKKLWGLKVLQELSQWTVRSIHDL-RFIS
           140             150        160        170        180

        200
chf.781   GGVAO

humcntf   SHQTGIPARGSHYIANNKKM
                 190        200
```

FIG._2

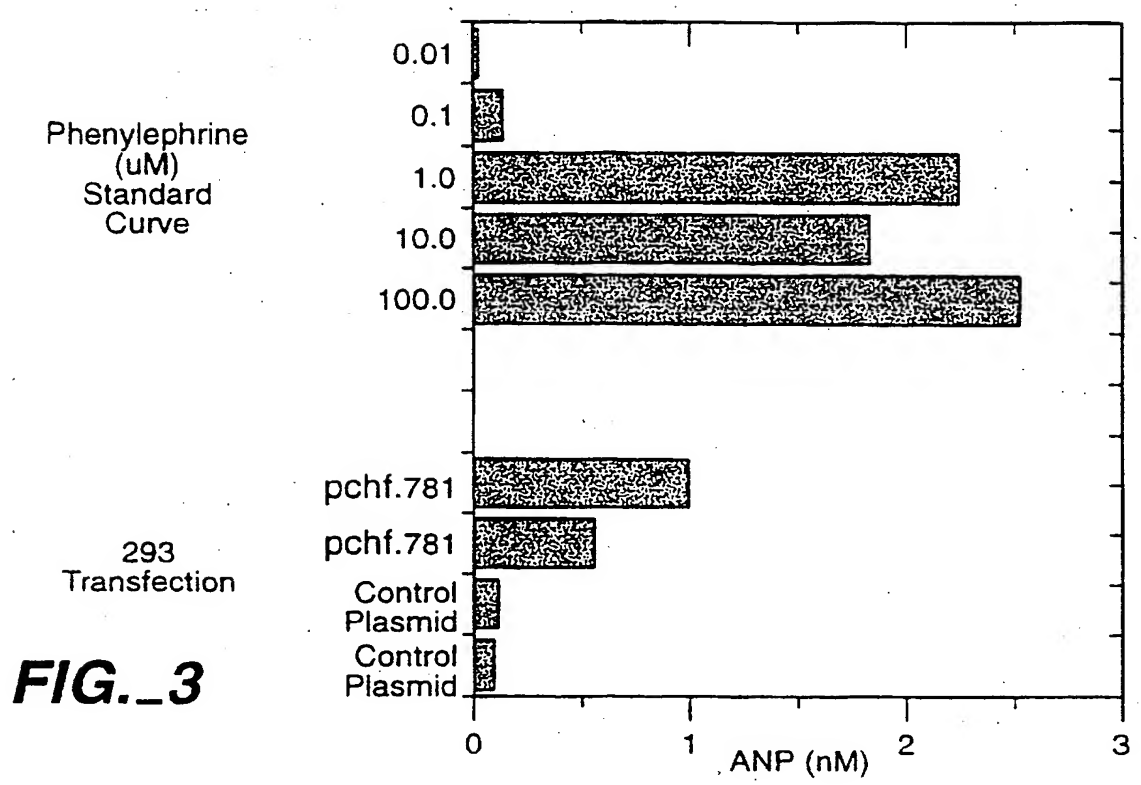
Phenylephrine (uM) Standard Curve
0.01
0.1
1.0
10.0
100.0
293 Transfection
pchf.781
pchf.781
Control Plasmid
Control Plasmid
0
1
2
3
ANP (nM)

FIG._3

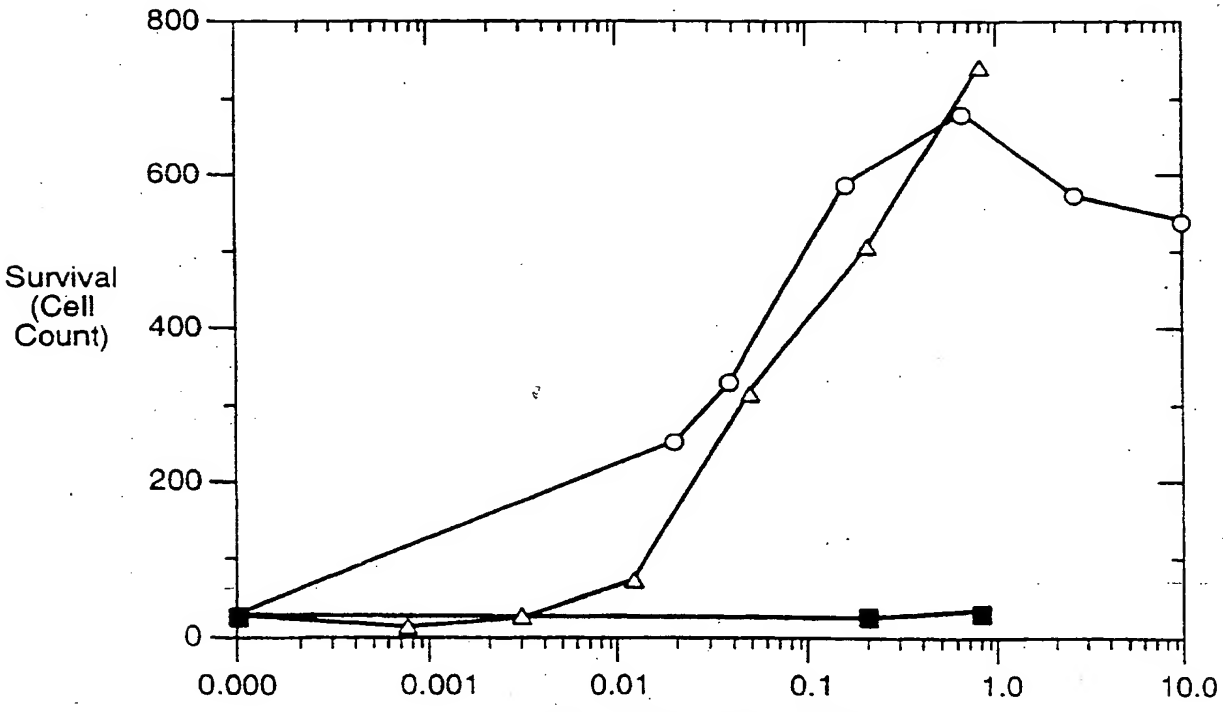
Survival (Cell Count)
800
600
400
200
0
0.000
0.001
0.01
0.1
1.0
10.0
CNTF Standard (ng/ml) or
Transfected 293 Conditioned Medium
(Fraction of Assay Vol)

FIG._4

```
   1  GTGAAGGGAG CCGGGATCAG CCAGGGGCCA GCATGAGCCG GAGGGAGGGA
      CACTTCCCTC GGCCCTAGTC GGTCCCCGGT CGTACTCGGC CTCCCTCCCT
   1                                      M  S  R   R  E  G

  51  AGTCTGGAAG ACCCCCAGAC TGATTCCTCA GTCTCACTTC TTCCCCACTT
      TCAGACCTTC TGGGGGTCTG ACTAAGGAGT CAGAGTGAAG AAGGGGTGAA
   7  S  L  E  D   P  Q  T   D  S  S   V  S  L  L   P  H  L

 101  GGAGGCCAAG ATCCGTCAGA CACACAGCCT TGCGCACCTC CTCACCAAAT
      CCTCCGGTTC TAGGCAGTCT GTGTGTCGGA ACGCGTGGAG GAGTGGTTTA
  24   E  A  K   I  R  Q  T   H  S  L   A  H  L   L  T  K  Y

 151  ACGCTGAGCA GCTGCTCCAG GAATATGTGC AGCTCCAGGG AGACCCCTTC
      TGCGACTCGT CGACGAGGTC CTTATACACG TCGAGGTCCC TCTGGGGAAG
  41    A  E  Q   L  L  Q   E  Y  V  Q   L  Q  G   D  P  F

 201  GGGCTGCCCA GCTTCTCGCC GCCGCGGCTG CCGGTGGCCG GCCTGAGCGC
      CCCGACGGGT CGAAGAGCGG CGGCGCCGAC GGCCACCGGC CGGACTCGCG
  57  G  L  P  S   F  S  P   P  R  L   P  V  A  G   L  S  A

 251  CCCGGCTCCG AGCCACGCGG GGCTGCCAGT GCACGAGCGG CTGCGGCTGG
      GGGCCGAGGC TCGGTGCGCC CCGACGGTCA CGTGCTCGCC GACGCCGACC
  74   P  A  P   S  H  A  G   L  P  V   H  E  R   L  R  L  D

 301  ACGCGGCGGC GCTGGCCGCG CTGCCCCCGC TGCTGGACGC AGTGTGTCGC
      TGCGCCGCCG CGACCGGCGC GACGGGGGCG ACGACCTGCG TCACACAGCG
  91    A  A  A   L  A  A   L  P  P  L   L  D  A   V  C  R

 351  CGCCAGGCCG AGCTGAACCC GCGCGCGCCG CGCCTGCTGC GCCGCCTGGA
      GCGGTCCGGC TCGACTTGGG CGCGCGCGGC GCGGACGACG CGGCGGACCT
 107  R  Q  A  E   L  N  P   R  A  P   R  L  L  R   R  L  E

 401  GGACGCGGCG CGCCAGGCCC GGGCCCTGGG CGCCGCCGTG GAGGCCTTGC
      CCTGCGCCGC GCGGTCCGGG CCCGGGACCC GCGGCGGCAC CTCCGGAACG
 124   D  A  A   R  Q  A  R   A  L  G   A  A  V   E  A  L  L

 451  TGGCCGCGCT GGGCGCCGCC AACCGCGGGC CCCGGGCCGA GCCCCCCGCC
      ACCGGCGCGA CCCGCGGCGG TTGGCGCCCG GGGCCCGGCT CGGGGGCGG
 141    A  A  L   G  A  A   N  R  G  P   R  A  E   P  P  A
```

FIG._5A

```
501  GCCACCGCCT CAGCCGCCTC CGCCACCGGG GTCTTCCCCG CCAAGGTGCT
     CGGTGGCGGA GTCGGCGGAG GCGGTGGCCC CAGAAGGGGC GGTTCCACGA
157  A  T  A  S   A  A  S   A  T  G   V  F  P  A   K  V  L

551  GGGGCTCCGC GTTTGCGGCC TCTACCGCGA GTGGCTGAGC CGCACCGAGG
     CCCCGAGGCG CAAACGCCGG AGATGGCGCT CACCGACTCG GCGTGGCTCC
174   G  L  R   V  C  G  L   Y  R  E   W  L  S   R  T  E  G

601  GCGACCTGGG CCAGCTGCTG CCCGGGGGCT CGGCCTGAGC GCCGCGGGGC
     CGCTGGACCC GGTCGACGAC GGGCCCCCGA GCCGGACTCG CGGCGCCCCG
191    D  L  G   Q  L  L   P  G  G  S   A  O

651  AGCTCGCCCC GCCTCCTCCC GCTGGGTTCC GTCTCTCCTT CCGCTTCTTT
     TCGAGCGGGG CGGAGGAGGG CGACCCAAGG CAGAGAGGAA GGCGAAGAAA

701  GTCTTTCTCT GCCGCTGTCG GTGTCTGTCT GTCTGCTCTT AGCTGTCTCC
     CAGAAAGAGA CGGCGACAGC CACAGACAGA CAGACGAGAA TCGACAGAGG

751  ATTGCCTCGG CCTTCTTTGC TTTTTGTGGG GGAGAGGGGA GGGGACGGGC
     TAACGGAGCC GGAAGAAACG AAAAACACCC CCTCTCCCCT CCCCTGCCCG

801  AGGGTCTCTG TCGCCCAGGC TGGGGTGCAG TGGCGCGATC CCAGCACTGC
     TCCCAGAGAC AGCGGGTCCG ACCCCACGTC ACCGCGCTAG GGTCGTGACG

851  AGCCTCAACC TCCTGGGCTC AAGCCATCCT TCCGCCTCAG CTTCCCCAGC
     TCGGAGTTGG AGGACCCGAG TTCGGTAGGA AGGCGGAGTC GAAGGGGTCG

901  AGCTGGGACT ACAGGCACGC GCCACCACAG CCGGCTAATT TTTTATTTAA
     TCGACCCTGA TGTCCGTGCG CGGTGGTGTC GGCCGATTAA AAAATAAATT

951  TTTTTTGTAG AGACGAGGTT TCGCCATGTT GCCCAGGCTG GTCTTGAACT
     AAAAAACATC TCTGCTCCAA AGCGGTACAA CGGGTCCGAC CAGAACTTGA

1001 CCGGGGCTCA AGCGATCC
     GGCCCCGAGT TCGCTAGG
```

FIG._5B

```
humctl    1 MSRREGSLEDPQTDSSVSLLPHLEAKIRQTHSLAHLLTKYAEQLLQEYVQLQG
            ** ******* ***** * ************** ** ********** **** **
chf.781   1 MSQREGSLEDHQTDSSISFLPHLEAKIRQTHNLARLLTKYAEQLLEEYVQQQG

humctl   54 DPFGLPSFSPPRLPVAGLSAPAPSHAGLPVHERLRLDAAALAALPPLLDAVCR
            ***** ******* **** ********** **** ***** ** ***** *
chf.781  54 EPFGLPGFSPPRLPLAGLSGPAPSHAGLPVSERLRQDAAALSVLPALLDAVRR

humctl  107 RQAELNPRAPRLLRRLEDAARQARALGAAVEALLAALGAANRGPRAEPPAATA
            ********** ******* ******** ******* *** **
chf.781 107 RQAELNPRAPRLLRSLEDAARQVRALGAAVETVLAALGAAARGPGPEPVTVAT

humctl  160 --SAASATGVFPAKVLGLRVCGLYREWLSRTEGDLGQLLPGGSA
              * *  * * ***** ***** ** ********** *** *
chf.781 160 LFTANSTAGIFSAKVLGFHVCGLYGEWVSRTEGDLGQLVPGGVA
```

FIG._6

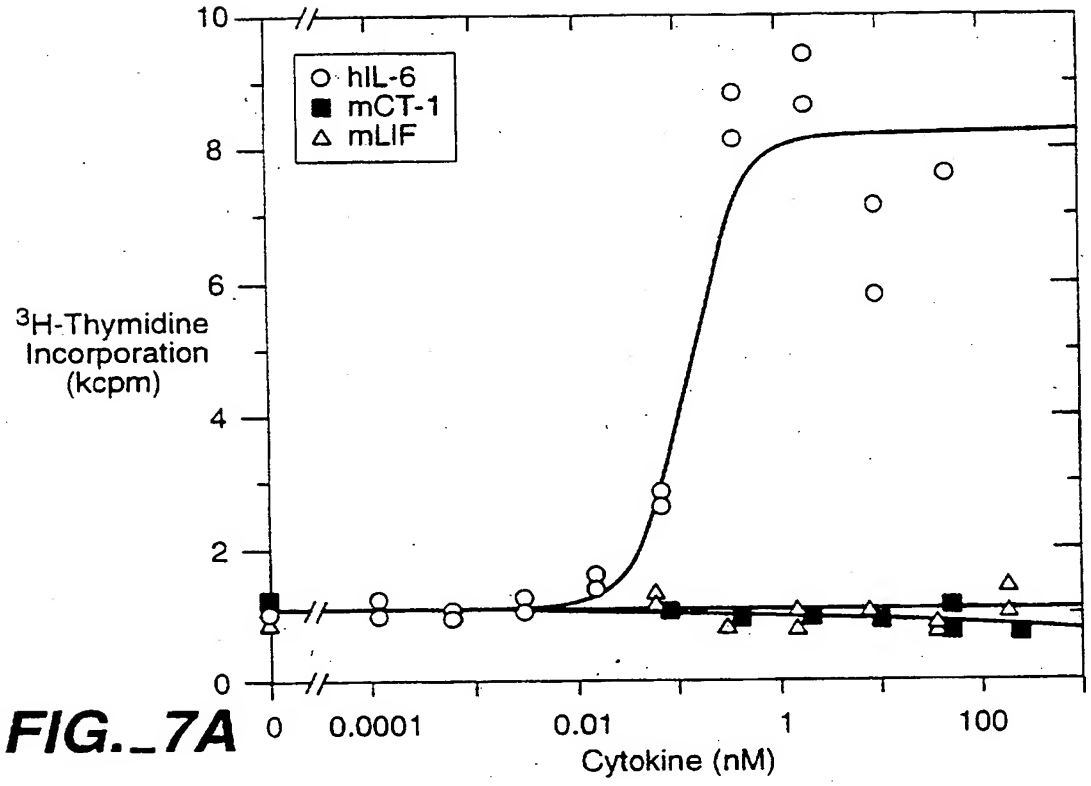
hIL-6
mCT-1
mLIF
^{3}H-Thymidine Incorporation (kcpm)
10
8
6
4
2
0
0
0.0001
0.01
1
100
Cytokine (nM)

FIG._7A

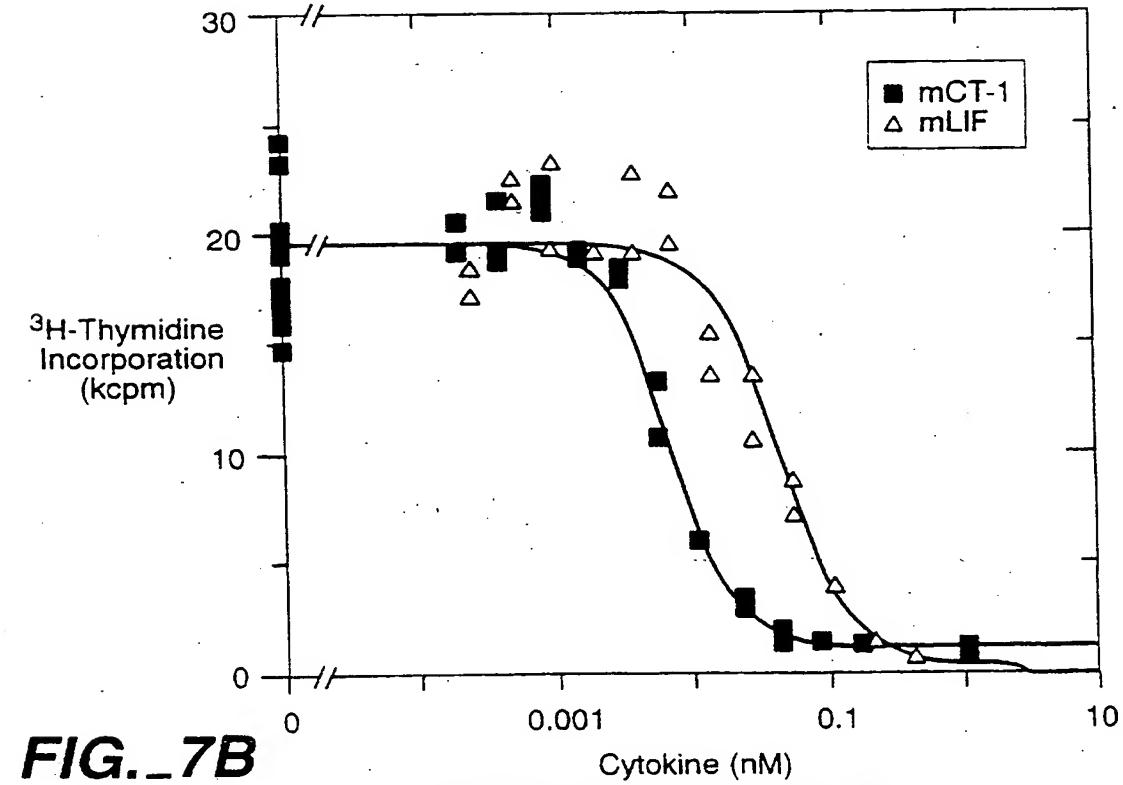
mCT-1
mLIF
^{3}H-Thymidine Incorporation (kcpm)
30
20
10
0
0
0.001
0.1
10
Cytokine (nM)

FIG._7B

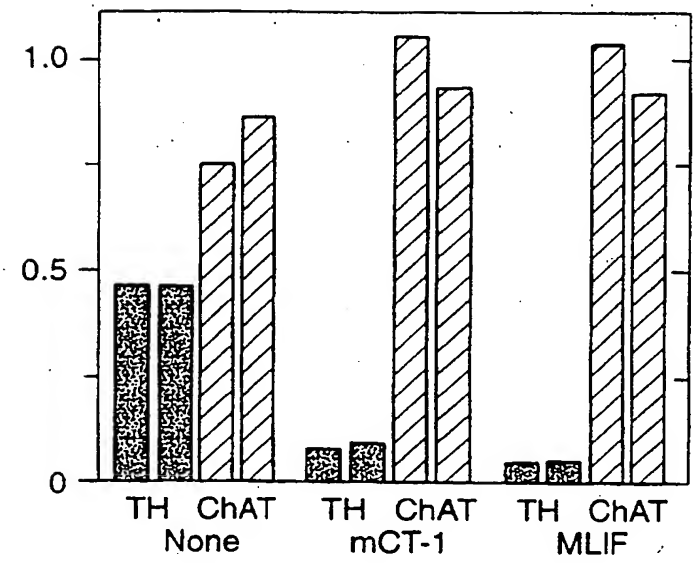

FIG._8A

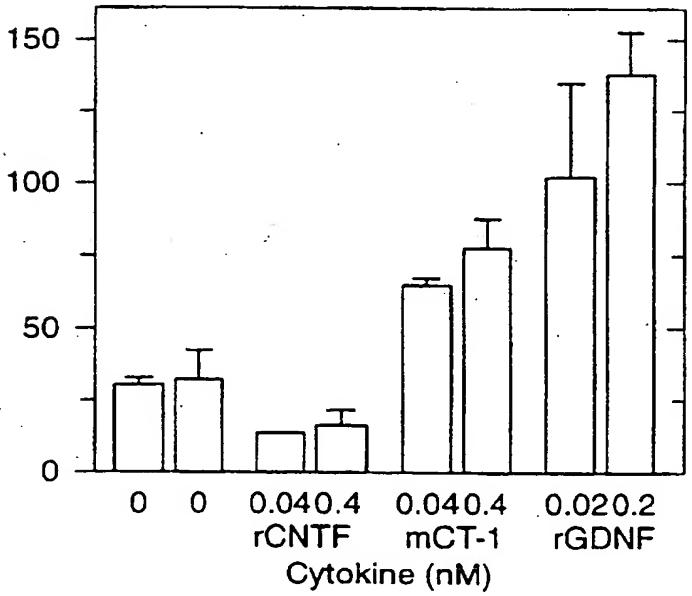

FIG._8B

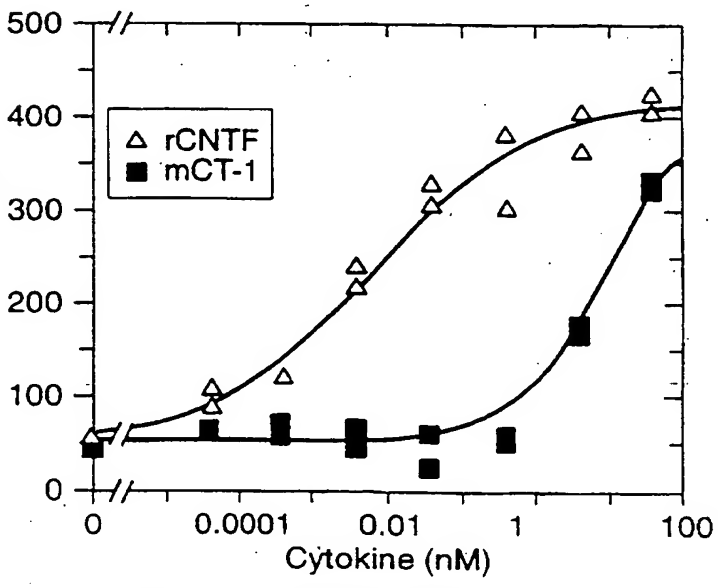

FIG._8C

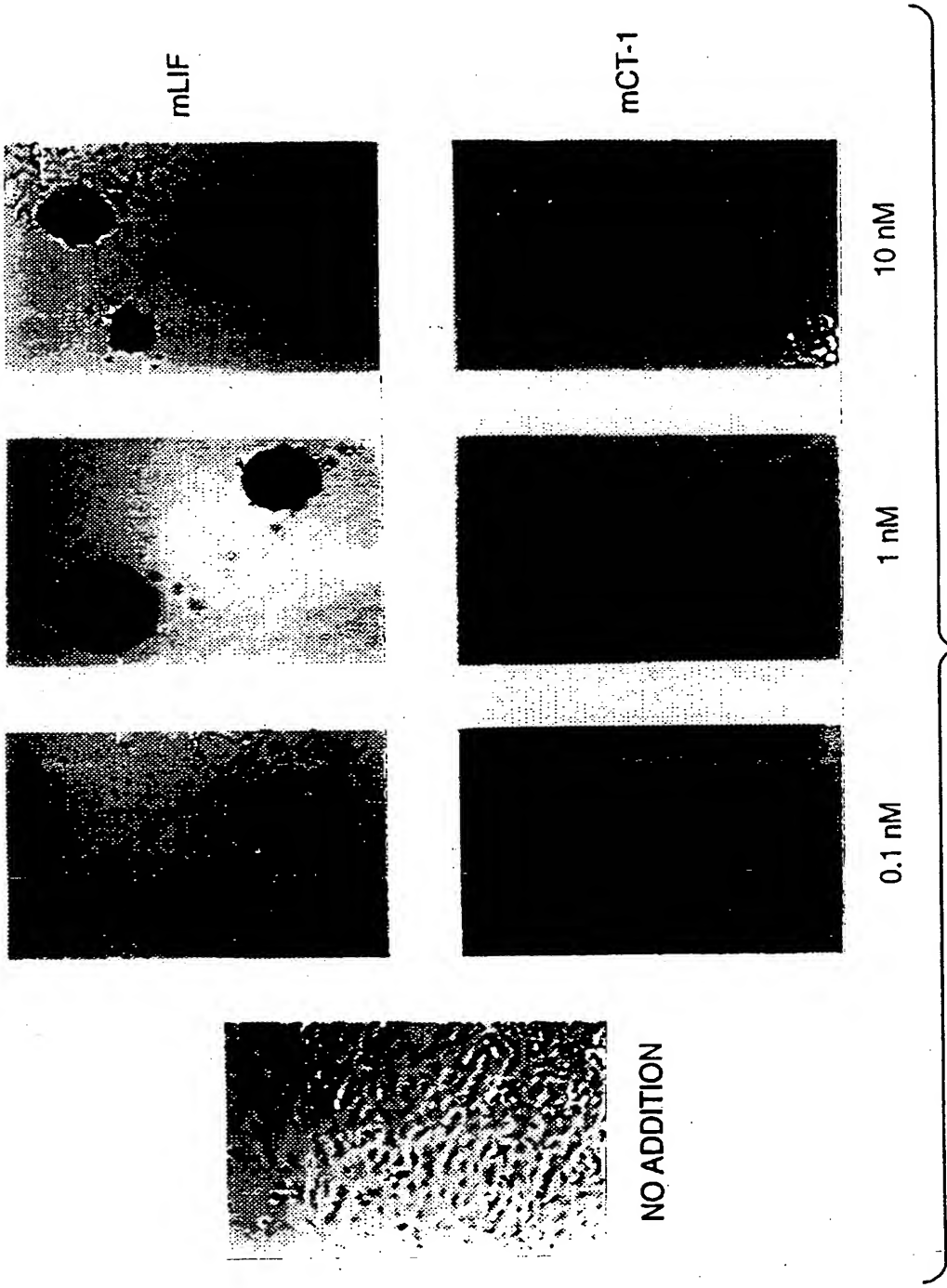

FIG._9

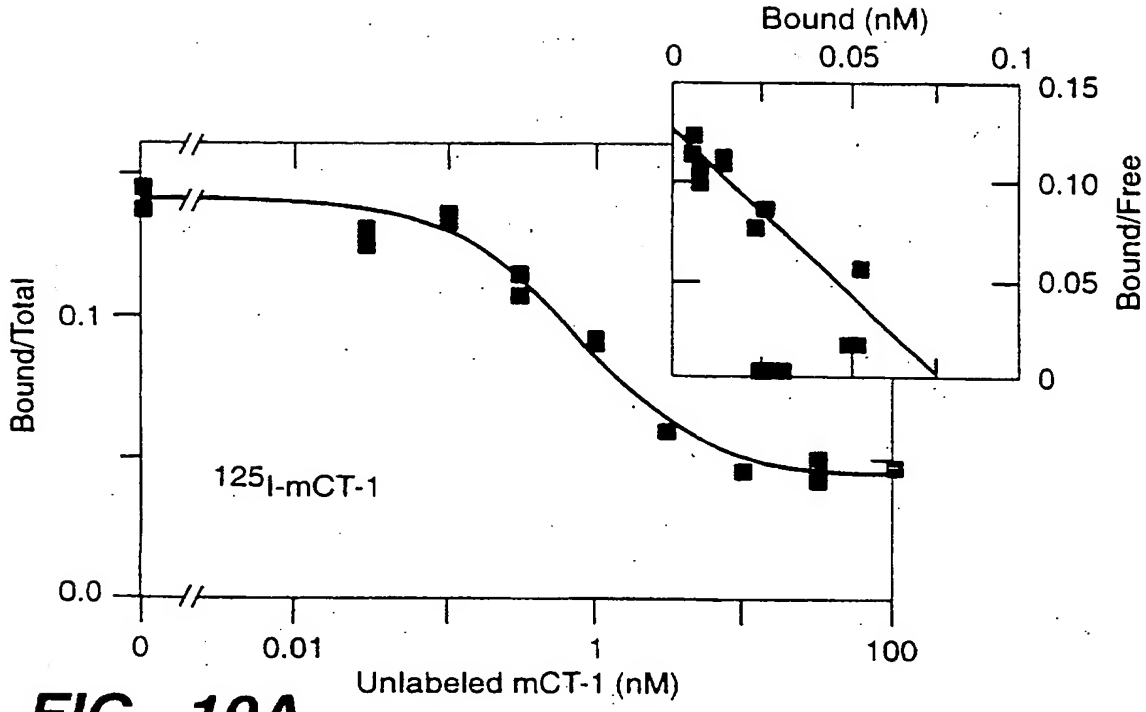

FIG._10A

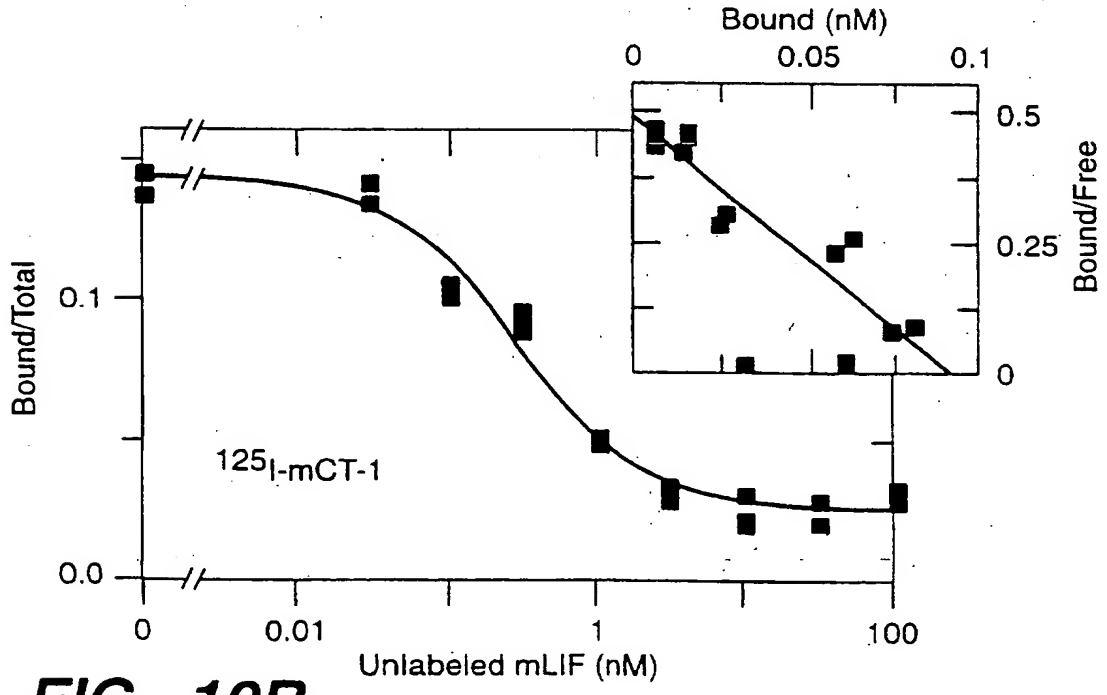

FIG._10B

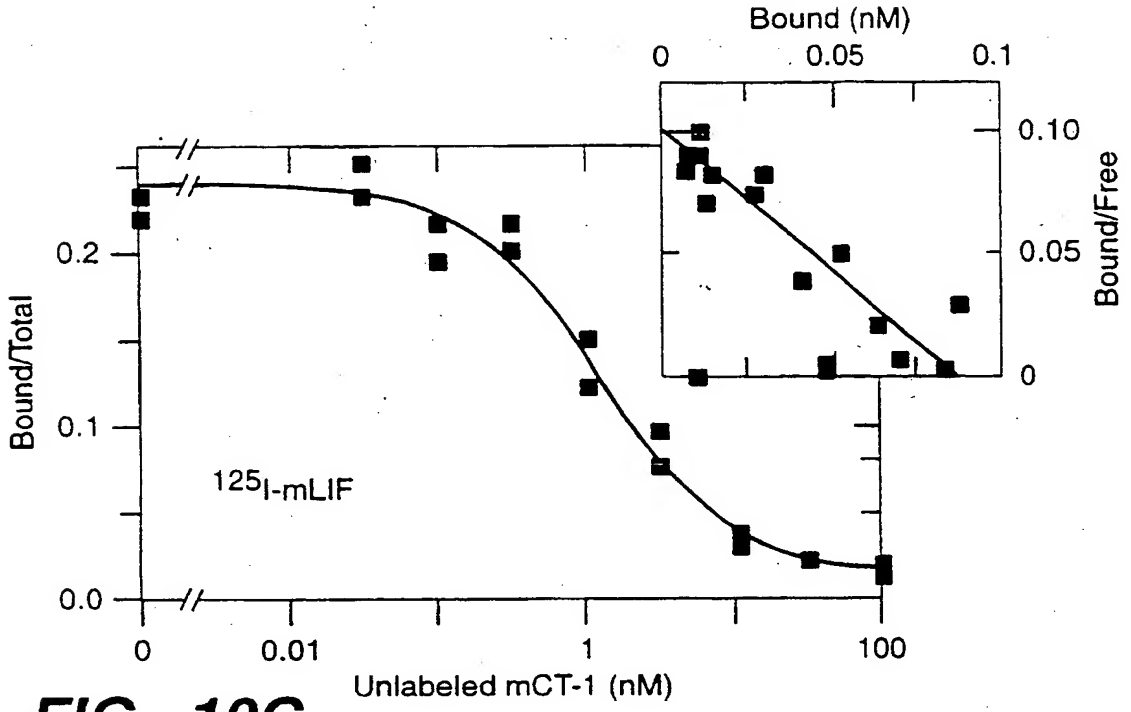

FIG._10C

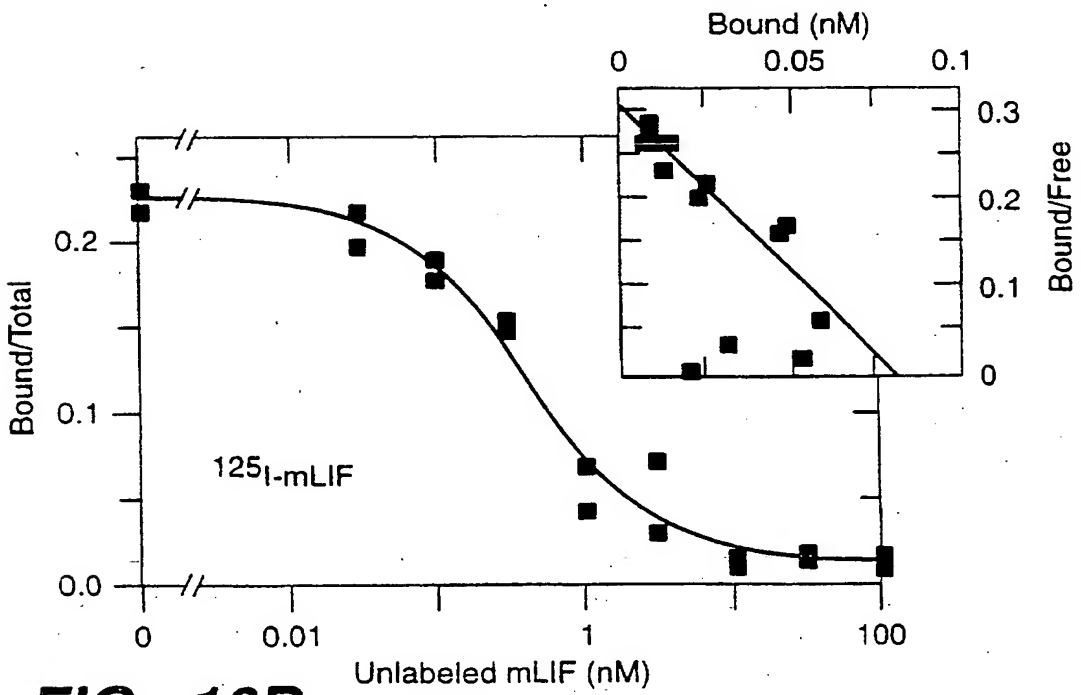

FIG._10D

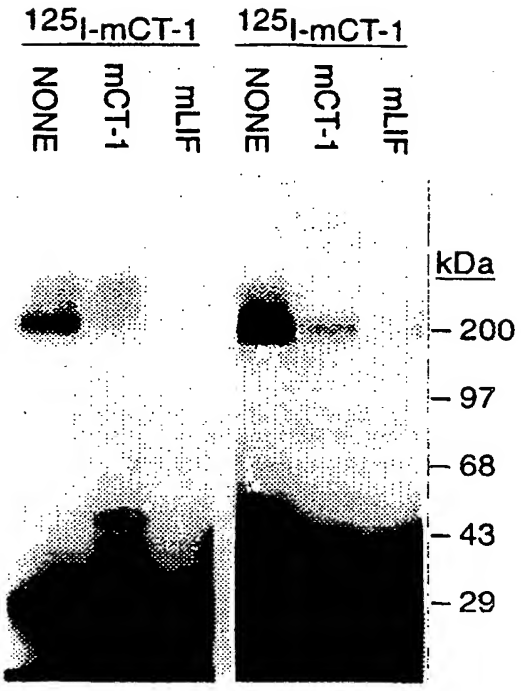

FIG._11

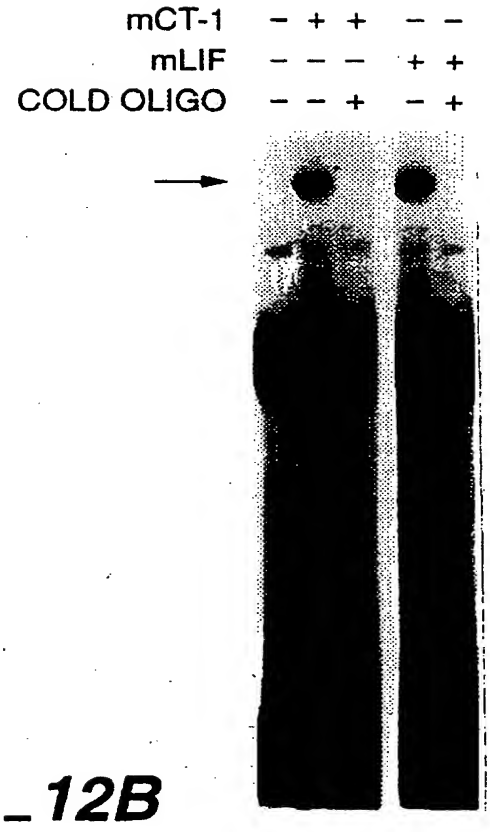

FIG._12B

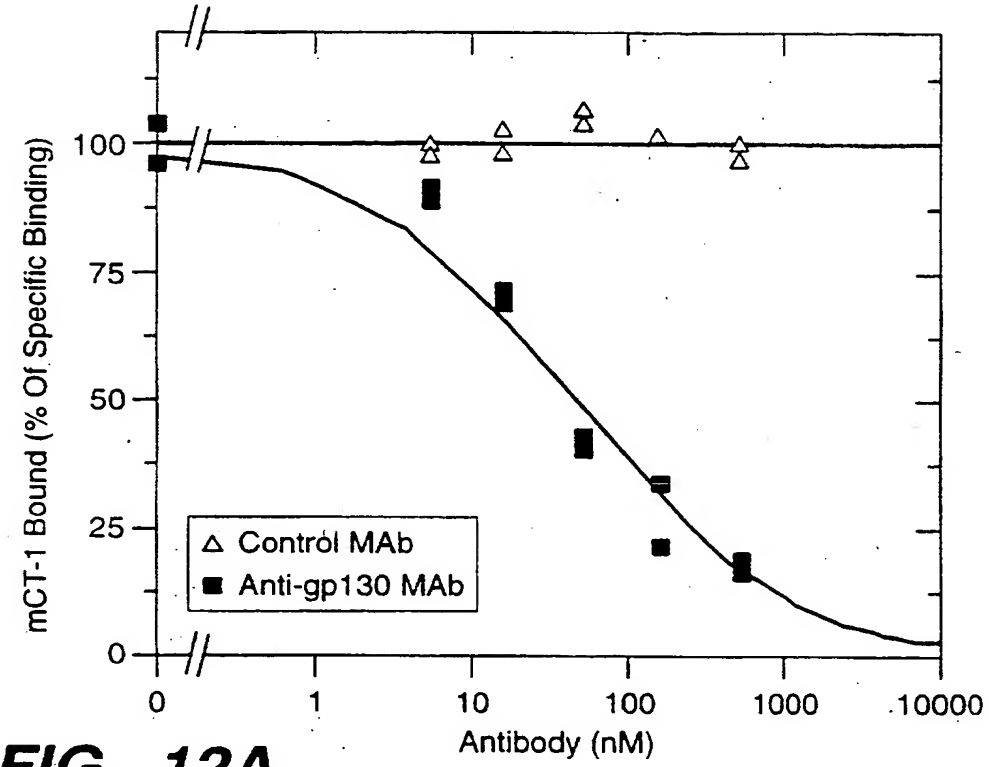

FIG._12A

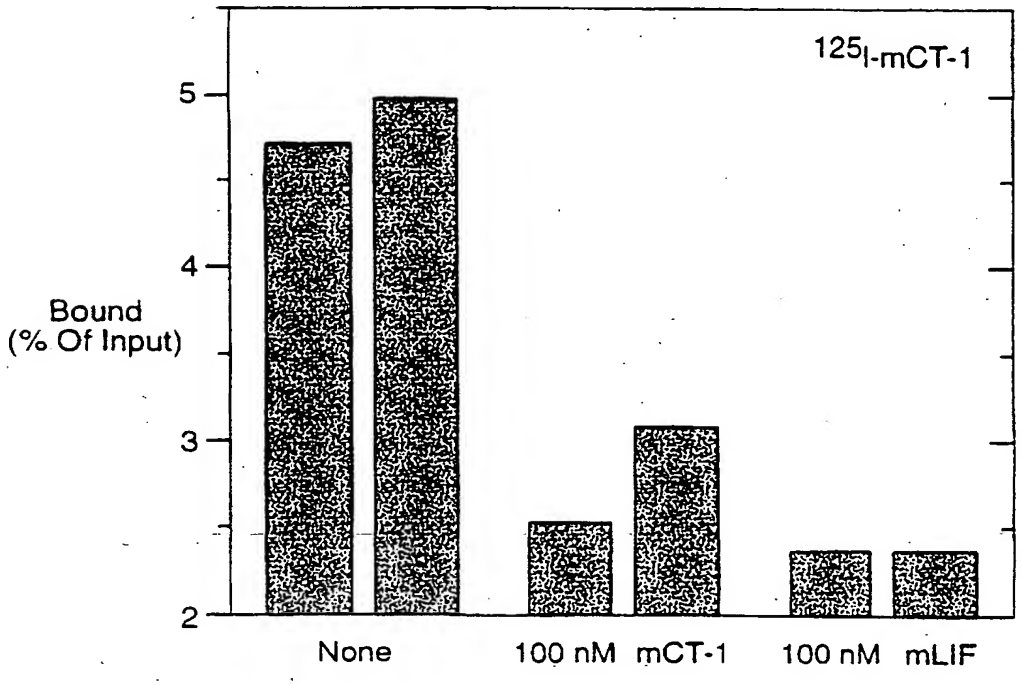

FIG._13A

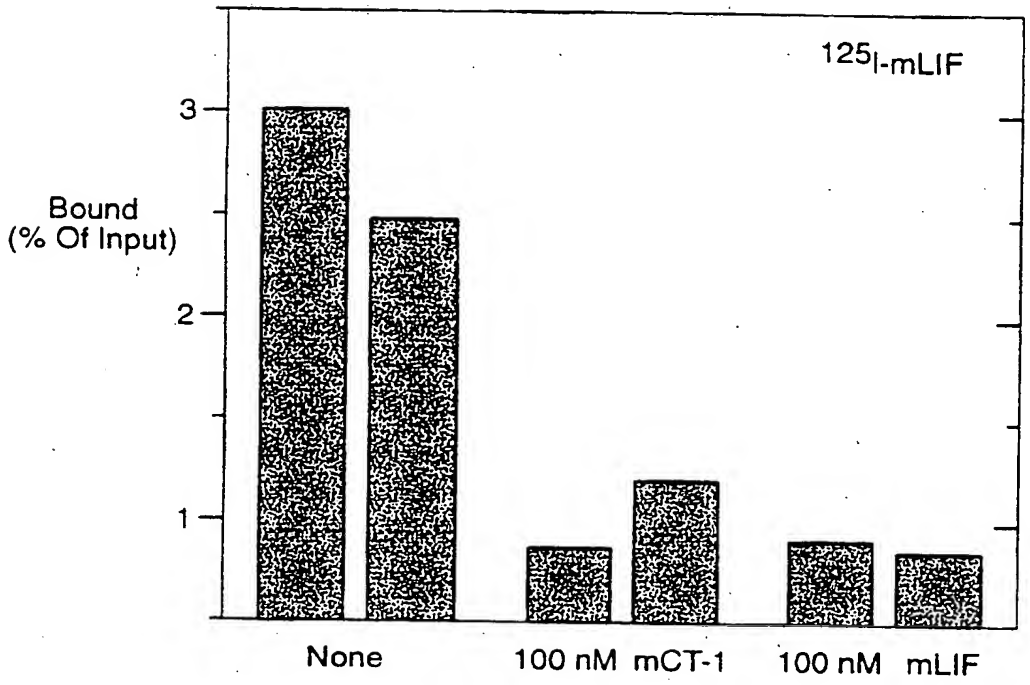

FIG._13B

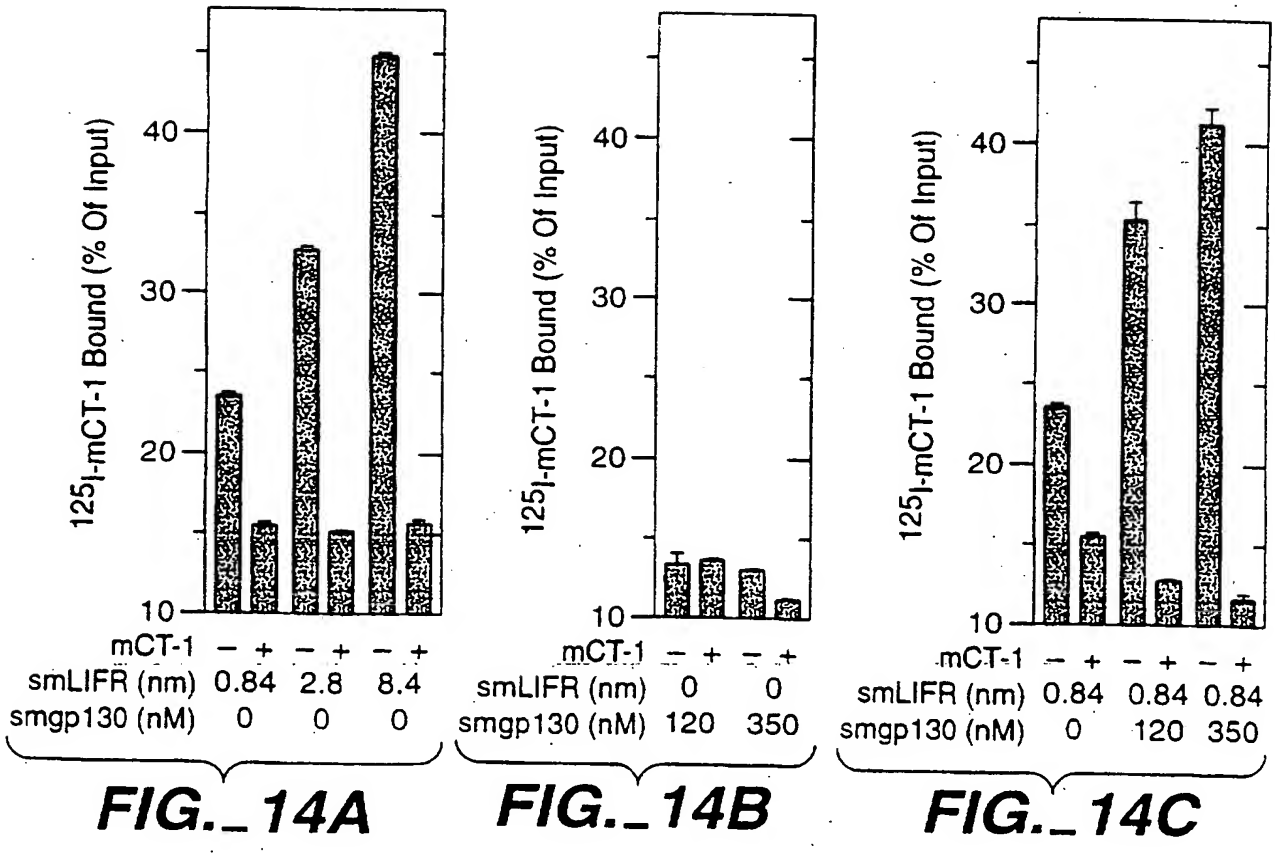

FIG._14A FIG._14B FIG._14C

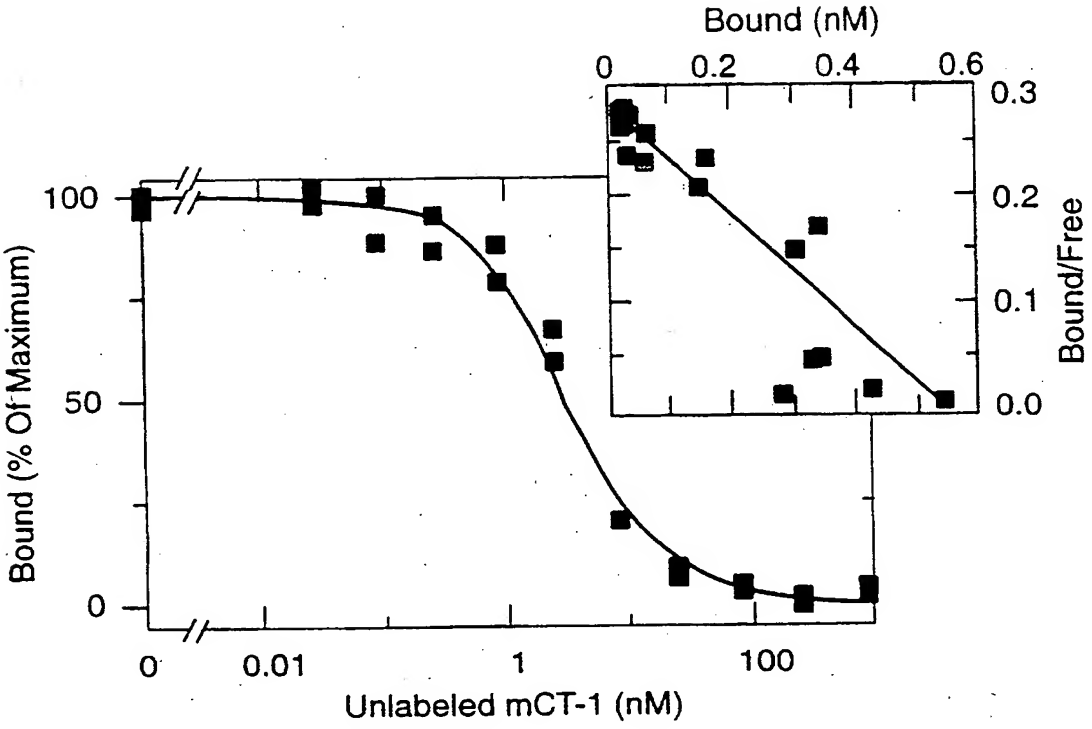

FIG._14D

mCT-1							
24	mLIF						
16	22	hOSM					
19	17	19	mCNTF				
26	18	23	46	cCNTF/ GPA			
19	18	23	18	21	mIL-11		
15	17	14	14	13	15	mIL-6	
79	80	100	82	43	88	41	Human HOMOLOG

FIG._15A

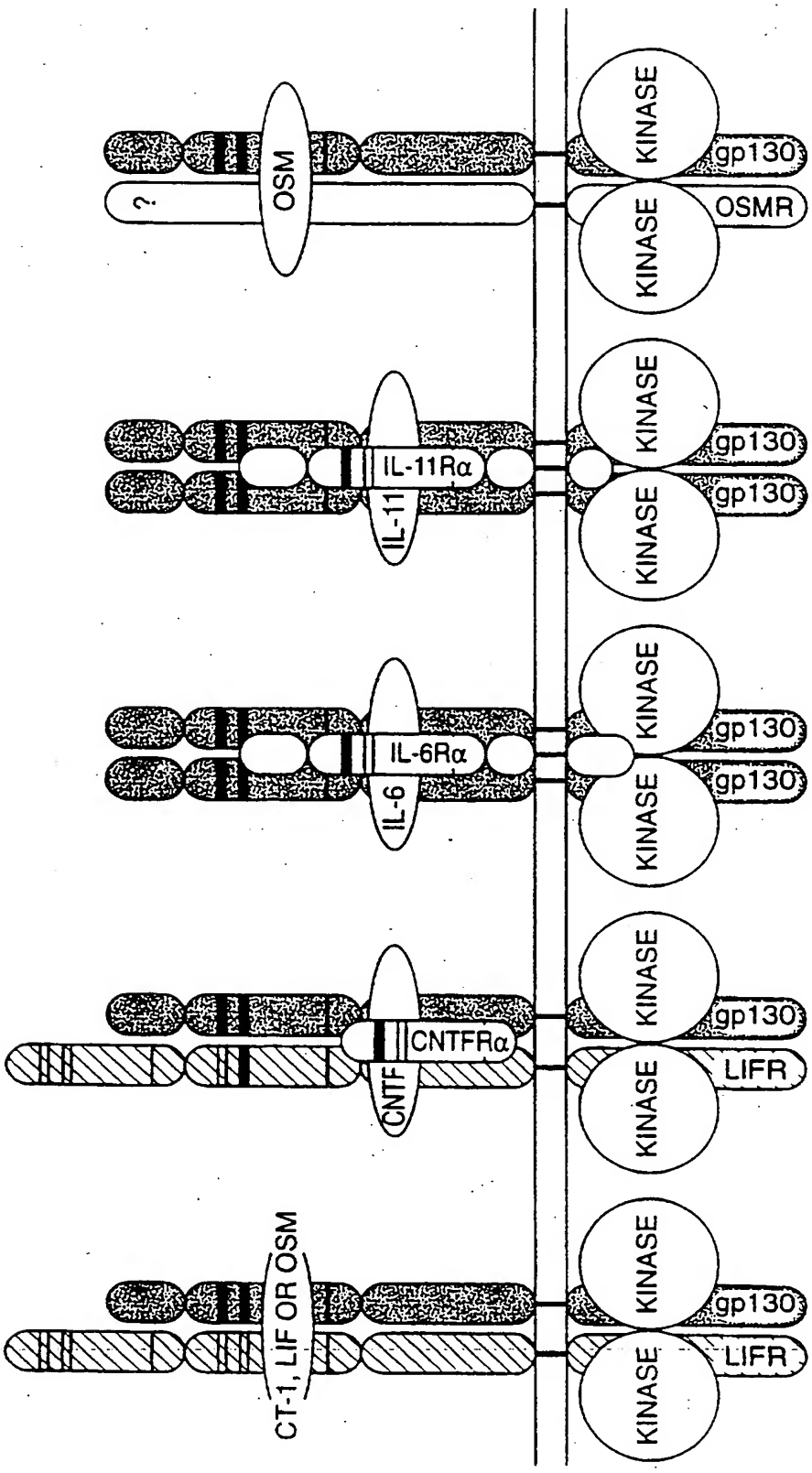
?
OSM
KINASE
gp130
KINASE
OSMR
IL-11Rα
IL-11
KINASE
gp130
KINASE
gp130
IL-6Rα
IL-6
KINASE
gp130
KINASE
gp130
CNTFRα
CNTF
KINASE
gp130
KINASE
LIFR
CT-1, LIF OR OSM
KINASE
gp130
KINASE
LIFR

FIG._15B

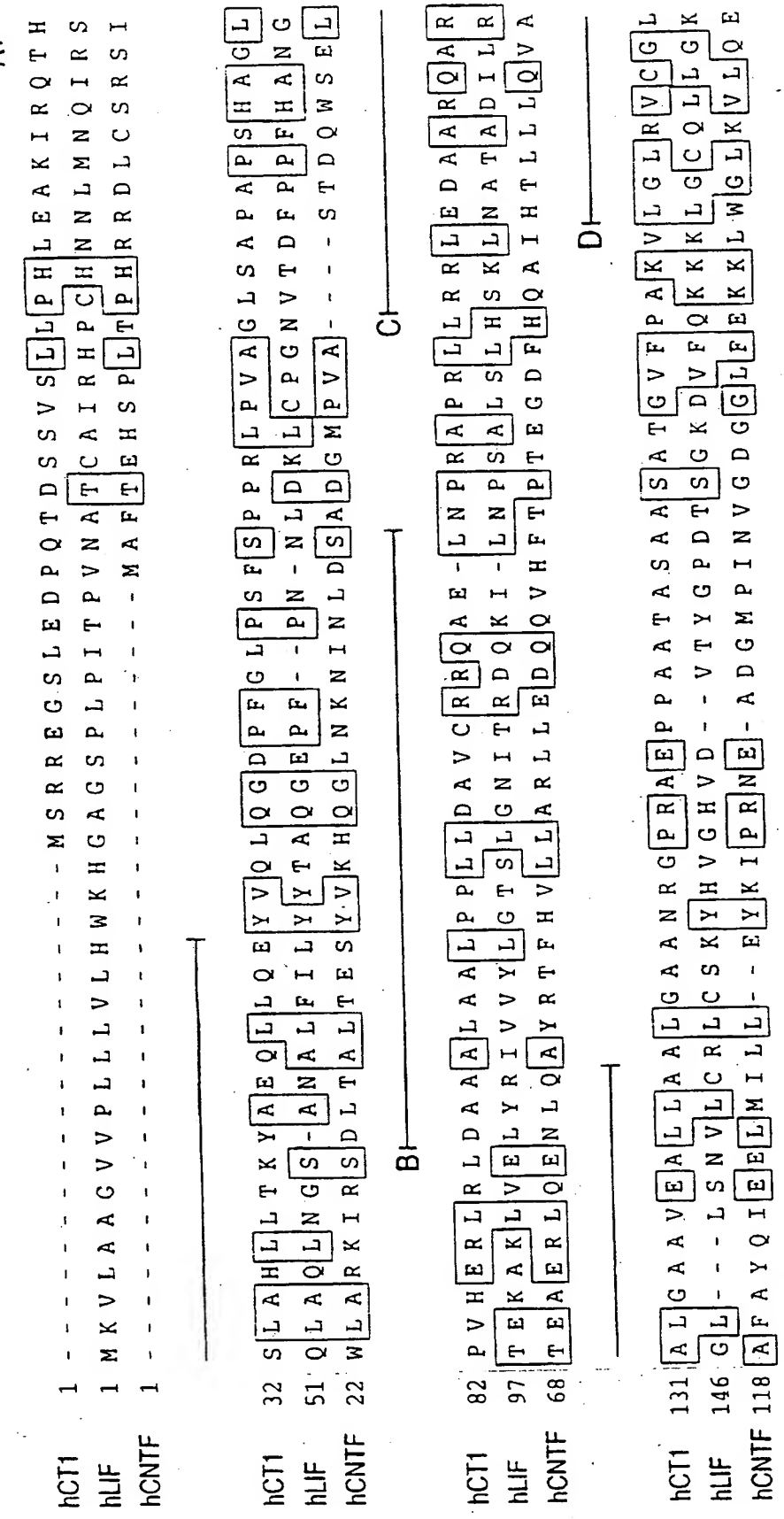

FIG._16

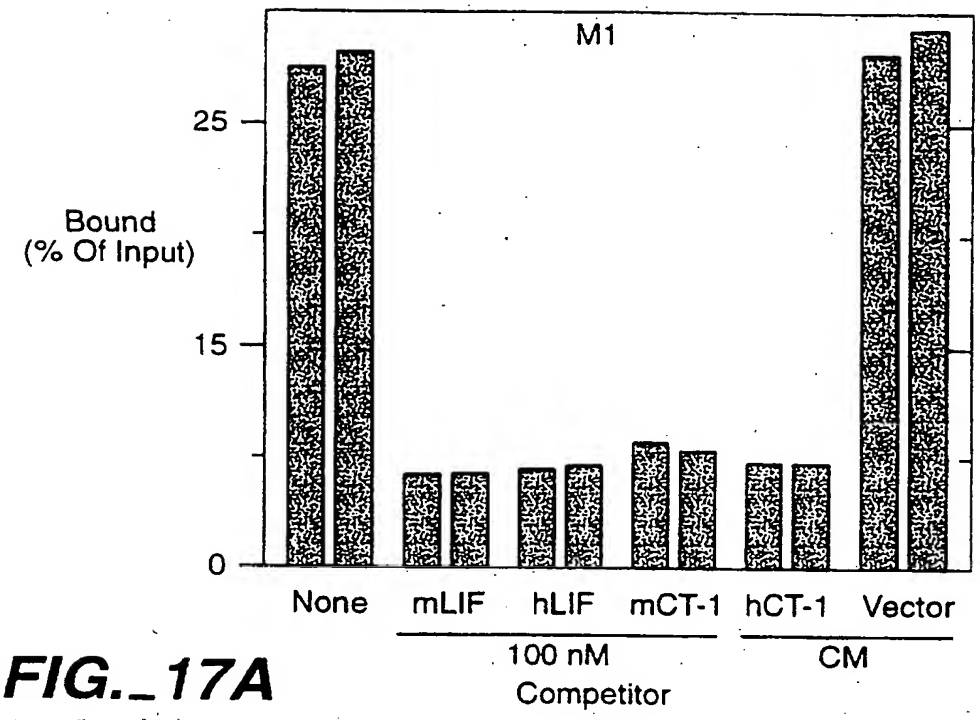

FIG._17A

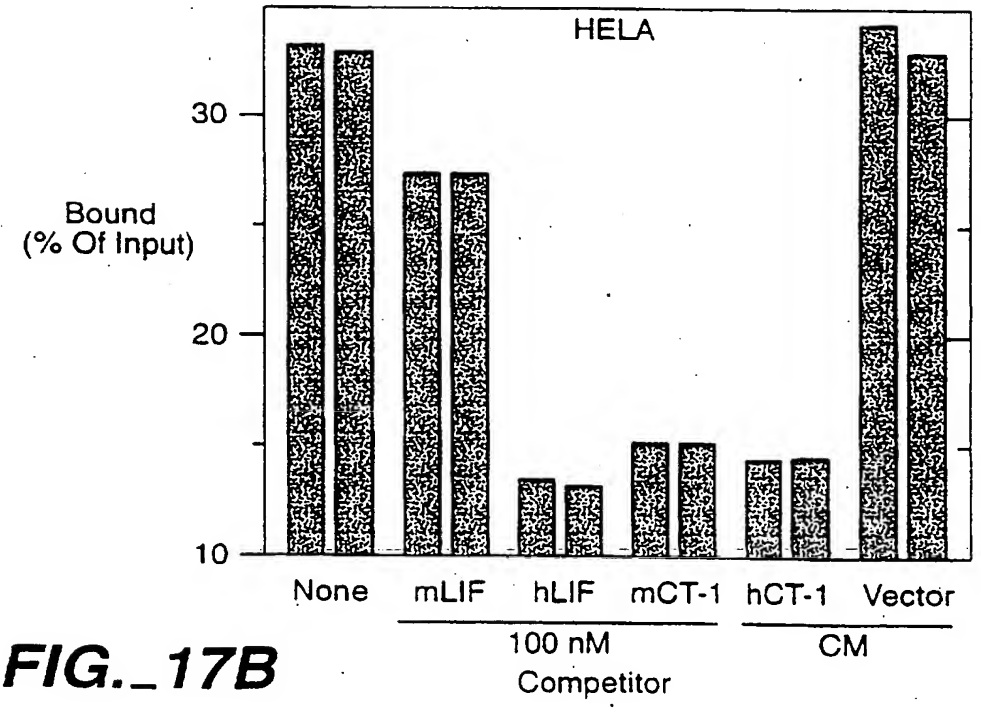

FIG._17B

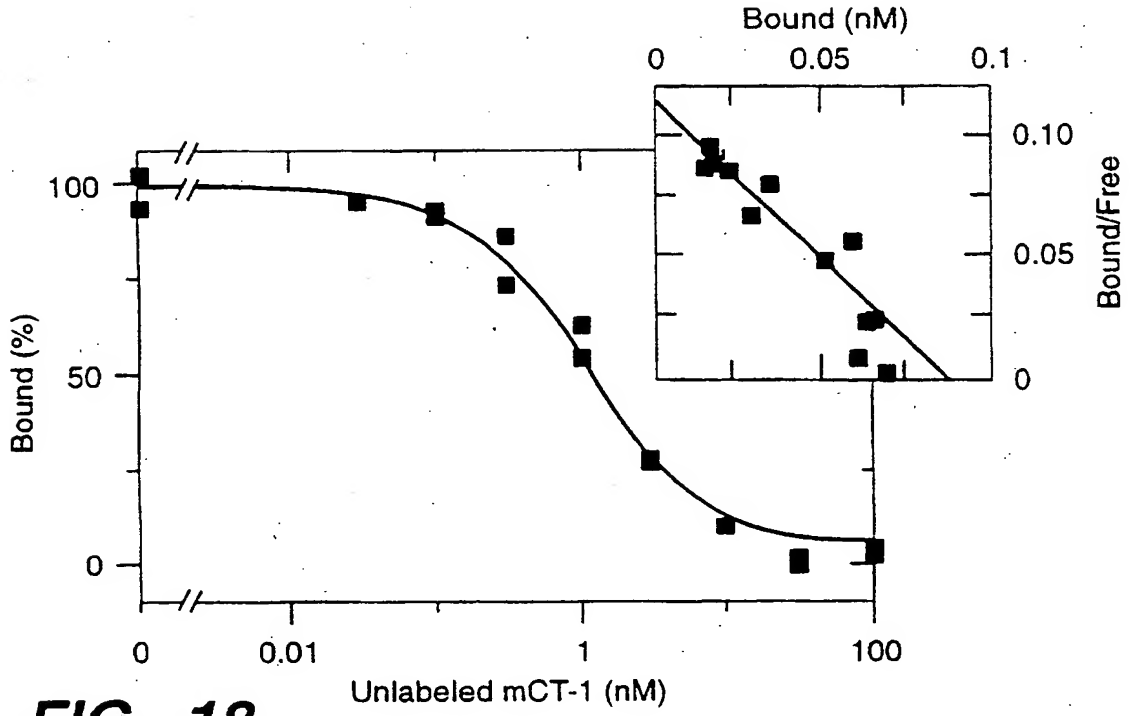
Bound (nM)
0
0.05
0.1
0.10
0.05
0
Bound/Free
100
50
0
Bound (%)
0
0.01
1
100
Unlabeled mCT-1 (nM)

FIG._18

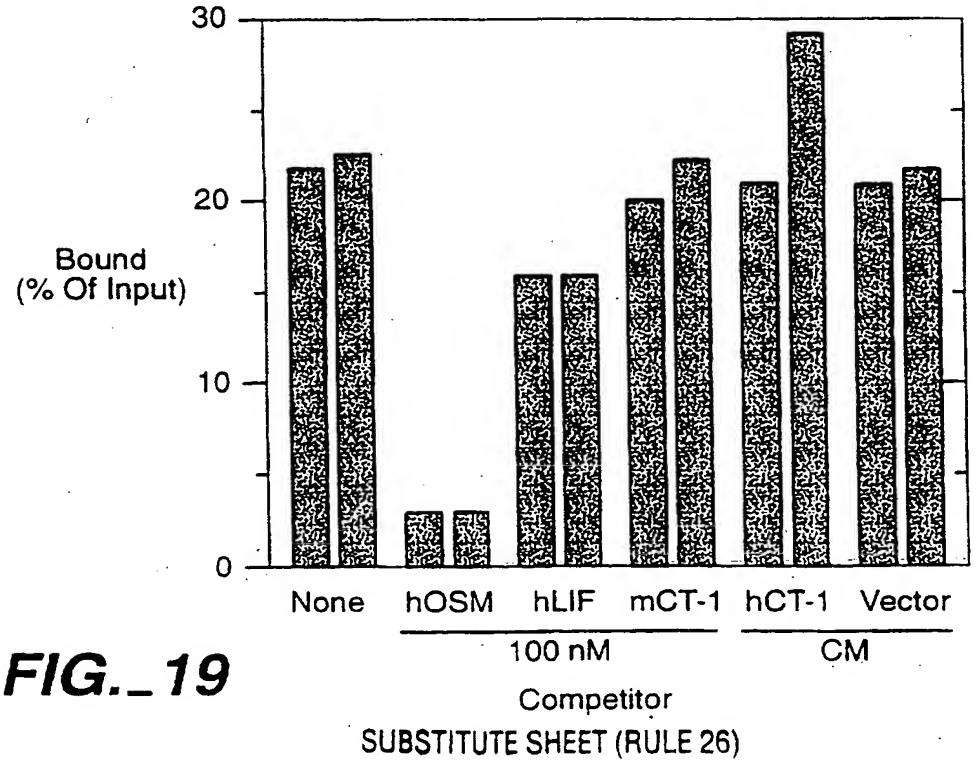
30
20
10
0
Bound
(% Of Input)
None
hOSM
hLIF
mCT-1
hCT-1
Vector
100 nM
CM
Competitor

FIG._19

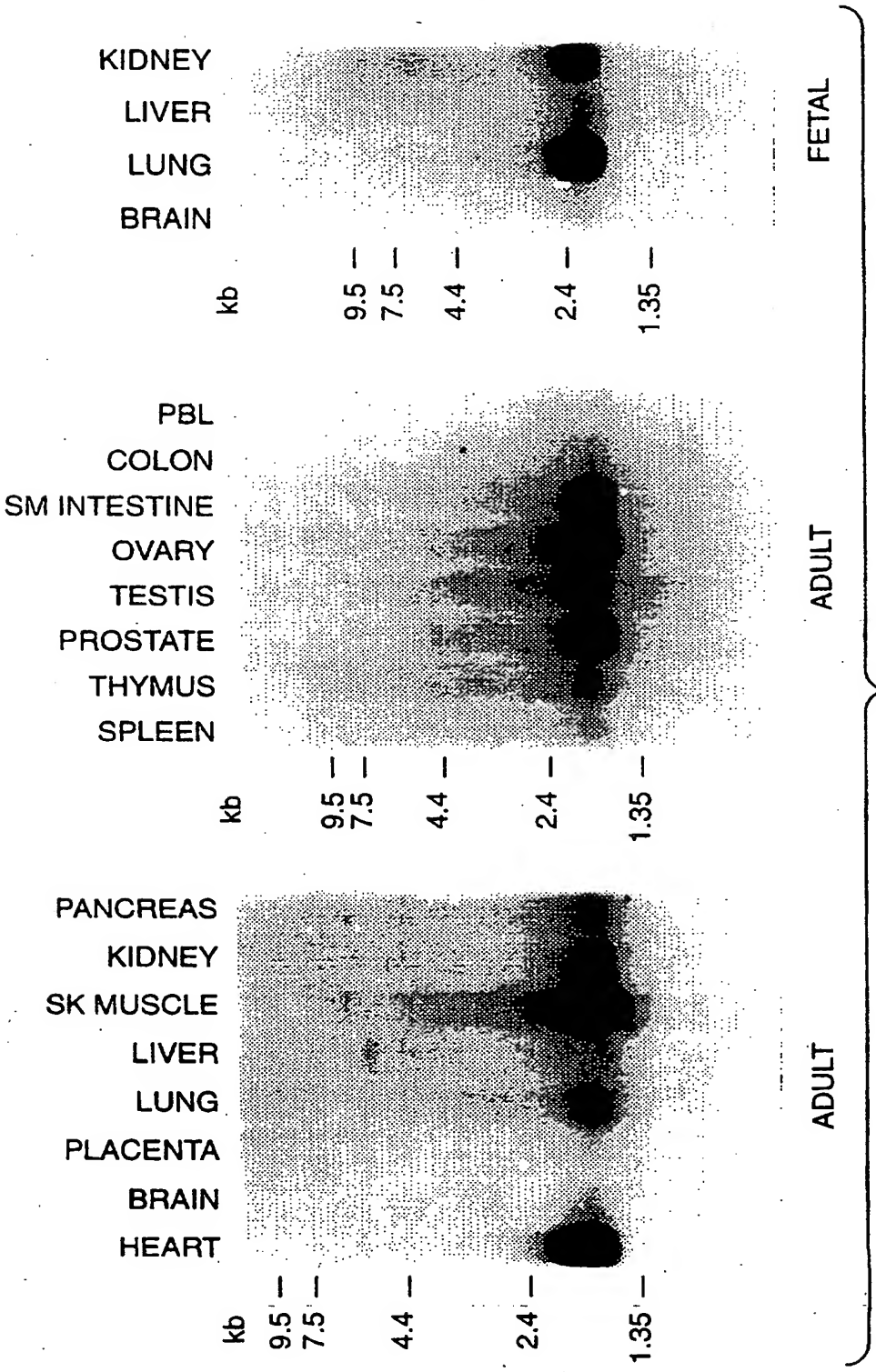
KIDNEY
LIVER
LUNG
BRAIN
kb
9.5
7.5
4.4
2.4
1.35
FETAL
PBL
COLON
SM INTESTINE
OVARY
TESTIS
PROSTATE
THYMUS
SPLEEN
kb
9.5
7.5
4.4
2.4
1.35
ADULT
PANCREAS
KIDNEY
SK MUSCLE
LIVER
LUNG
PLACENTA
BRAIN
HEART
kb
9.5
7.5
4.4
2.4
1.35
ADULT

FIG._20

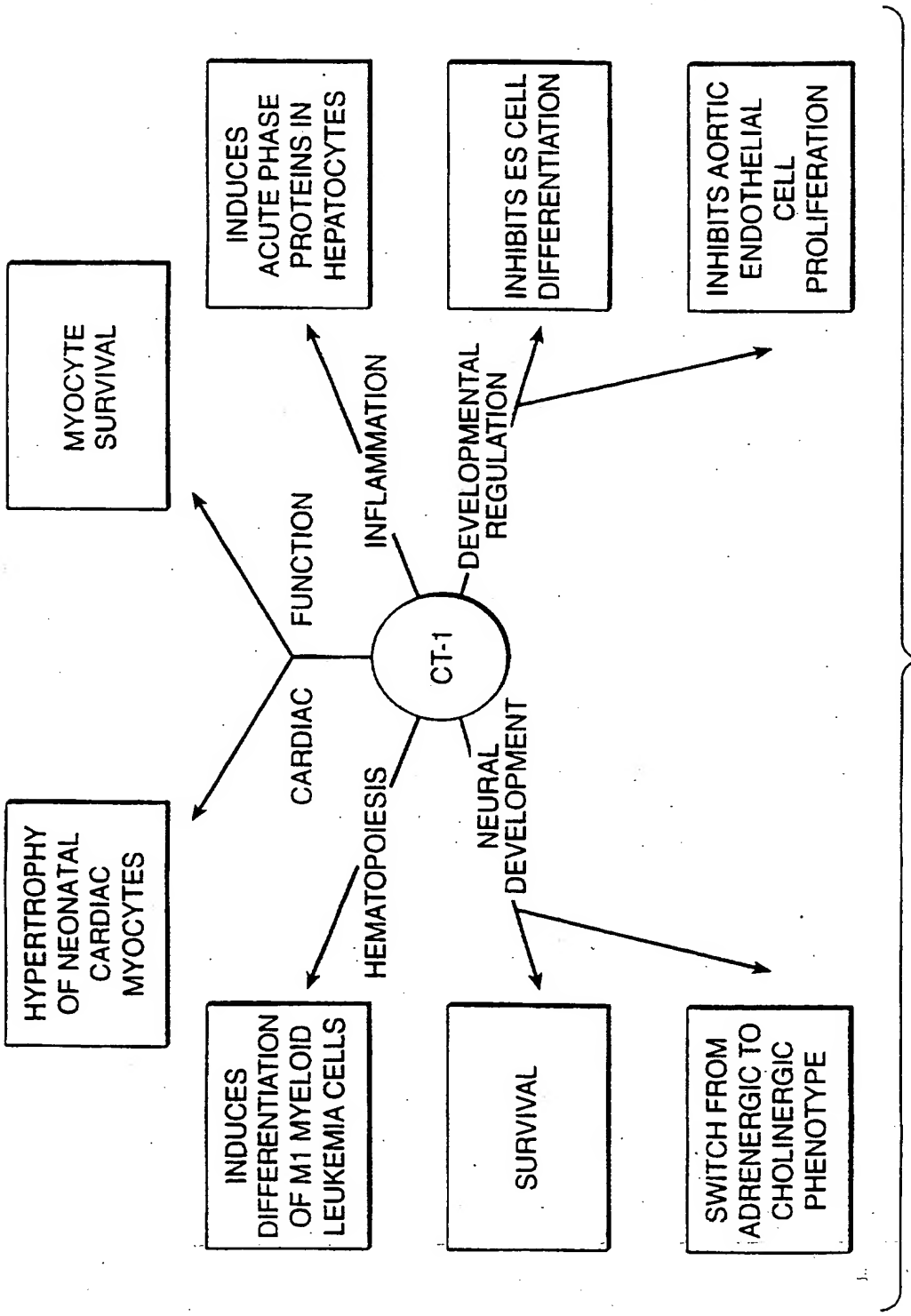
CT-1
FUNCTION
CARDIAC
INFLAMMATION
DEVELOPMENTAL REGULATION
HEMATOPOIESIS
NEURAL DEVELOPMENT
MYOCYTE SURVIVAL
HYPERTROPHY OF NEONATAL CARDIAC MYOCYTES
INDUCES ACUTE PHASE PROTEINS IN HEPATOCYTES
INHIBITS ES CELL DIFFERENTIATION
INHIBITS AORTIC ENDOTHELIAL CELL PROLIFERATION
INDUCES DIFFERENTIATION OF M1 MYELOID LEUKEMIA CELLS
SURVIVAL
SWITCH FROM ADRENERGIC TO CHOLINERGIC PHENOTYPE

FIG._21

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/06, 5/08, A61K 38/19, A01K 67/02

A3

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08/601,395 14 February 1996 (14.02.96) US

(71) Applicants: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US). THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).

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(74) Agents: TORCHIA, Timothy, E. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
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(54) Title: CARDIOTROPHIN AND USES THEREFOR

(57) Abstract

Isolated CT-1, isolated DNA encoding CT-1, and recombinant or synthetic methods of preparing CT-1 are disclosed. CT-1 is shown to bind to and activate the receptor, LIFRβ. These CT-1 molecules are shown to influence hypertrophic activity, neurological activity, and other activities associated with receptor LIFRβ. Accordingly, these compounds or their antagonists may be used for treatment of heart failure, arrhythmic disorders, inotropic disorders, neurological disorders, and other disorders associated with the LIFRβ.

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SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
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US	United States of America
UZ	Uzbekistan
VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Interna[illegible] Application No
PCT/US 97/02675

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/08 A61K38/19 A01K67/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29237 A (GENENTECH INC) 2 November 1995 cited in the application see page 61, line 3 - page 65, line 2 -----	1-25

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1997	26.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

tional application No.
PCT/US 97/ 02675

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. [X] Claims Nos.: 15,16,19-25
because they relate to subject matter not required to be searched by this Authority, namely

Remark: Although claim(s) 15,16,19-25 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos..
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern: Application No
PCT/US 97/02675

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9529237 A	02-11-95	US 5534615 A	09-07-96
		US 5571893 A	05-11-96
		AU 2635395 A	16-11-95
		CA 2188017 A	02-11-95
		EP 0755446 A	29-01-97
		US 5624806 A	29-04-97
		US 5627073 A	06-05-97
		US 5571675 A	05-11-96